University of Alberta

LEPTIN REGULATION OF GLUCOSE HOMEOSTASIS

by

NI THUYEN LAM ©

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LIST OF ABBREVIATIONS

Abreviation	Description
Adβ-gal	adenovirus expressing β-galactosidase
AdPTP1B	adenovirus expressing PTP1B
AMPK	5'-AMP-activated protein kinase
AGRP	agouti-related peptide
ASO	antisense oligonucleotide
β-gal	β-galactosidase
BMI	body mass index
$[Ca^{2+}]_i$	intracellular calcium concentration
CAMK	Ca^{2+}/calmodulin-dependent protein kinase
cAMP	cyclic AMP
CART	cocaine- and amphetamine-regulated transcript
CHO	chinese hamster ovary
Cy3	iodocarbocyanine
DIO	diet-induced obesity
DMEM	Dulbecco's modified Eagle's medium
DMH	dorsal medial hypothalamus
DMSO	dimethylsulfoxide
2-DOG	2-deoxyglucose
ECL	enhanced chemiluminescence
EDL	extensor digitorum longus
ERK2	extracellular signal-regulated kinase 2
FBS	fetal bovine serum
Glc-6-Pase	glucose-6-phosphatase
GLP-1	glucagon-like peptide-1
GLUT	glucose transporter
HF	high fat
HGP	hepatic glucose production
HNF	hepatocyte nuclear factor
HRP	horseradish peroxidase
ICV	intracerebroventricular
IGF-1	insulin-like growth factor-1
IP	intraperitoneal
IR	insulin receptor
IRS	insulin receptor substrate
IV	intravenous
ITT	insulin tolerance test

JAK	janus kinase
K_{ATP}	ATP-sensitive K^+
LC-CoA	long-chain fatty acyl CoA
LF	low fat
LH	lateral hypothalamus
MAPK	mitogen-activated protein kinase
MEM	minimal essential media
MI	metabolic inhibition
MODY	maturity-onset diabetes of the young
3-*O*-MG	3-*O*-methylglucose
NPY	neuropeptide Y
ObR	leptin receptor
OGTT	oral glucose tolerance test
PBS	phosphate-buffered saline
PEPCK	phosphoenolpyruvate carboxylase
PDE	phosphodiesterase
PDH	pyruvate dehydrogenase complex
PFU	plaque forming units
PI3K	phosphatidylinositol-3-kinase
PKB	protein kinase B
PPAR	peroxisome proliferators-activated receptor
POMC	proopiomelanocortin
PTP	protein tyrosine phosphatase
PTP1B	protein tyrosine phosphatase 1B
pTyr	phosphotyrosine
SHP2	Src homology 2 containing phosphatase 2
SOCS	suppressors of cytokine signalling proteins
STAT	signal transducers and activators of transcription
SREBP-1	sterol regulatory element binding protein-1
TZD	thiazolidinediones
UCP	uncoupling protein
UTR	untranslated region
VDCC	voltage-dependent Ca^{2+} channel
VMH	ventral medial hypothalamus

CHAPTER 1
INTRODUCTION

1.1. Obesity and Diabetes

Diabetes mellitus, once considered to be a disease found primarily in developed countries, has since progressed into possibly the next global health epidemic (1). In 2000, it was estimated that over 177 million people worldwide had diabetes (2). By 2010 and 2025, this number is proposed to rise to 220 million and over 300 million respectively (2-5). In Canada alone, 1.2 - 1.4 million people representing 4.9 - 5.8% of the population over the age of 12 reportedly had diabetes in 1999 (6). The prevalence is drastically higher in the aging population with approximately 12% of the population over the age of 65 estimated to have had diabetes in 2001 (7). It is estimated that 1 out of every 3 men and 2 out of every 5 women born in the United States in 2000 will be diagnosed with diabetes mellitus (8). Diabetes and its complications were calculated to cost the Canadian healthcare system between $4.756 and $5.23 billion U.S. dollars in 1998 (9). With the rising increase in obesity and in particular adolescent obesity (10), these figures are expected to escalate dramatically, creating an unwelcome burden on the healthcare budget. Prevention of diabetes is a key issue because of the huge premature morbidity and mortality associated with this disease and its complications (11-15).

Diabetes mellitus is characterized by an inability of the pancreatic β cells to produce insulin in the proper amount and time frame that is required for maintaining glucose homeostasis (16, 17). Insulin is the primary hormonal regulator of fuel metabolism, and its production and subsequent secretion from pancreatic β cells is caused by the absorption of glucose and other nutrients from the intestine into the circulation. Insulin acts on organs such as skeletal muscle, liver, and adipose tissue to stimulate the uptake of glucose and to store calories predominantly in the form of glycogen and fat. There are several classifications of diabetes mellitus, of which two, type 1 and type 2 diabetes, dominate in prevalence. One genetic subgroup of diabetes, maturity-onset diabetes of the

young (MODY), is characterized by a monogenetic defect that is acquired through an autosomal dominant mode and manifests in β cell dysfunctions before the age of 25 (18). Defects in any one of six genes (glucokinase, hepatocyte nuclear factor (HNF)-1α, HNF-4α, HNF-1β, insulin promoter factor 1, and NeuroD1(β2)) result in pancreatic β cell failure and hyperglycemia causing early-onset, non-insulin dependent diabetes. Gestational diabetes mellitus, or impaired glucose intolerance first diagnosed during pregnancy, affects approximately 4% of pregnant women a year and is a risk factor for development of type 2 diabetes in the mother (19). Type 1 diabetes results from autoimmune-mediated destruction of pancreatic β cells that produce insulin, leading to drastic deficiency or absence of insulin production. People with type 1 diabetes must take insulin in order to utilize glucose and to prevent ketoacidosis, a potentially life-threatening complication stemming from the inability to utilize blood glucose for energy. The combination of insulin deficiency and increased counterregulatory hormones leads to the release of free fatty acids into the circulation from adipose tissue which gets oxidized to ketone bodies (ß-hydroxybutyrate and acetoacetate) by the liver, resulting in ketonemia and metabolic acidosis. Often presenting at an early age, type 1 diabetes is currently more common than type 2 diabetes in children. However, with the increasing prevalence of type 2 diabetes in children and adolescents (20-22), this trend may see a change in the near future. While people with type 2 diabetes also present with impaired insulin secretion, this form of the disease has traditionally been characterized by the failure of target organs to respond normally to the action of insulin. As a result, glucose is not taken up by tissues and the excess circulating glucose in the blood leads to well-known complications of diabetes such as hypertension, cardiovascular disease, retinopathy and kidney failure. The identification of diabetes-specific autoimmunity in some adults and children with typical type 2 diabetes has given rise to a separate category know as type 1.5 or latent autoimmune diabetes of adults (23). Individuals predisposed to developing type 2 diabetes may be classified with impaired fasting glucose or impaired glucose tolerance. An

elevated fasting plasma glucose concentration is associated with increased hepatic glucose output and a defect in early insulin secretion whereas peripheral insulin resistance is characteristic of elevated plasma glucose levels 2 h following an oral glucose tolerance test (24). Type 2 diabetes accounts for over 90% of diabetes globally, and its increasing prevalence has been the major driving force in the diabetes epidemic in both developed and developing countries. As this form of diabetes will be the focus of this thesis, the term "diabetes" will henceforth refer to type 2 diabetes mellitus.

1.2. Obesity Sets the Stage for Diabetes

Largely linked to excessive caloric intake and decreased physical activity, the increasing prevalence of diabetes parallels an alarming increase in obesity worldwide. Body mass index (BMI; height/weight^2 in m/kg^2) is one measurement that has been used as an index of body fatness. Cut-off values of BMI have been established to classify people as overweight (BMI>25) and obese (BMI>30). According to this definition, approximately 15% of Canadians were estimated to be obese in 2002 (25), and the prevalence of adolescent obesity is escalating dramatically (10, 26). Obesity is associated with a significant increase in morbidity and mortality and thus is considered a major health issue (27). Approximately 80% of type 2 diabetic patients are overweight or obese, suggesting that obesity may have a significant role to play in the pathogenesis of diabetes (28-30). Increasing body fat is accompanied by profound changes in physiologic functions, contributing to the development of hyperlipidemia, hypertension, and insulin resistance. Insulin resistance manifests in decreased insulin stimulation of glucose uptake in muscle and fat and uncontrolled hepatic glucose output (31) while selectively increasing lipid uptake and deposition in non-adipose tissue (32). Fatty acid overload of myocardium, skeletal muscle, and pancreatic islets further potentiates lipotoxic heart disease, insulin resistance, and diabetes (32). Insulin resistance is a fundamental aspect of diabetes and is also linked to other pathophysiological conditions including hyperlipidemia,

atherosclerosis, and hypertension. The cluster of syndromes associated with insulin resistance is known as metabolic syndrome or syndrome X (33).

Although modifications in food consumption can result in weight loss, greater than 90% of individuals who lose weight by dieting eventually return to their original body weight (34). This suggests that biologic factors may predetermine an individual's body weight, and this weight is maintained through physiological responses that resist change. Lipostasis is a theory that has evolved based on this concept, and it proposes that a homeostatic mechanism exists that allows an individual's weight to remain relatively stable over time (35). Weight gain or loss results in concerted changes in energy consumption and expenditure that resists the initial changes. A genetic component predisposes an individual to attaining the ideal body weight, as determined by twin studies, analyses of familial aggregation, adoption studies and genetic analyses of susceptible populations, which concluded that obesity results from genetic factors (36-39). While genetics are important in the predisposition to obesity, studies of traditional populations that have become "westernized" (40, 41) provide evidence that environmental influences such as diet and reduced physical activity weigh heavily in the rise in obesity prevalence. However, in most developed countries where food is relatively abundant and lifestyle is sedentary, not all people become obese and not all obese individuals develop diabetes. The likelihood is that genetic factors lay the blueprints for susceptibility to obesity and diabetes and that interactions of these genes with environmental variables determine the translation to disease. These gene-environment interactions that have likely evolved with societal changes have transformed obesity from a cosmetic and social issue into a major public health concern.

Despite the fact that obesity is rampantly making its presence felt on the world stage, history tells us that food availability has often been scarce, a fact that is still reality in many parts of the world today. The view of man as hunter/gatherer chronically searching for food and intermittently finding success questions the relevance of a gene designed to prevent energy storage.

Evolutionary pressures may have selected traits that promote efficient storage of energy when food is available. The concept of the thrifty gene hypothesis, introduced in the 1960s (42), notes that the ability to efficiently store energy during periods of feasting confers a reproductive advantage to societies subjected to periods of famine. It was proposed that insulin promotes storage of nutrients when food is abundant and hypothesized that insulin resistance would develop over time when food supplies are easily obtainable, thus predicting the onset of insulin resistance with increasing obesity. The thrifty gene hypothesis has been adapted to include a belief that insulin resistance in skeletal muscle confers an advantage in times of food scarcity, thereby limiting hypoglycemia by reducing glucose uptake and the subsequent use of glucose by skeletal muscle (43). Chronic implementation of this protective response in the presence of food abundance may thus have contributed to the pathophysiology that we now know as diabetes. Insulin resistance, therefore, may have initially evolved as a physiological response to acute periods of overfeeding.

1.3. Adipose Tissue as an Endocrine Organ

Adipose tissue plays a critical role in the regulation of insulin sensitivity, as well as glucose and lipid metabolism. Once thought to serve solely as the storage facility for excess nutrients, a role for adipose tissue as an endocrine organ has evolved over the last decade (44). The role of fat in the regulation of glucose and lipid homeostasis is evident in animal and human models of lipodystrophy (45, 46). The loss of subcutaneous and visceral adipose tissue results in insulin resistance, hyperglycemia, dyslipidemia and hepatic steatosis. In the absence of adipose tissue, excess calories cannot be diverted to normal fat storage depots, and thus accumulate as triglyceride stores in liver, skeletal muscle, cardiac muscle and pancreatic islets. Abnormal intracellular triglyceride accumulation results in impaired insulin secretion and action leading to diabetes. Adipose tissue releases free fatty acids and a number of factors collectively termed adipocytokines that act in autocrine, paracrine and endocrine fashion to regulate different metabolic

processes (47). Several factors that have been implicated in the development of insulin resistance include tumour necrosis factor α, adiponectin, adipsin, acylation stimulation protein, interleukin-6 and resistin (47, 48). The hormone leptin, also secreted from adipose tissue, is now recognized to be a key regulator of energy homeostasis.

1.4. Parabiosis Studies Leading up to Discovery of Leptin

In 1953, the lipostatic theory of body weight control was put forth by Kennedy, which stated that body size is regulated via a hormone released from adipose tissue (35). It was postulated that a circulating molecule from adipose tissue stores communicates the state of nutritional abundance. A number of early parabiotic experiments, in which the circulatory systems of two animals were surgically connected, examined the physiological effects when blood contents were exchanged between animals. Cross circulation between obese mice with lesions in the ventral medial hypothalamus (VMH) and normal, non-lesioned rats led to feeding suppression and weight loss in the non-lesioned rats (49). These findings suggested that the non-lesioned rats were responsive to a circulating factor produced by the lesioned rats, but the lesioned rats were insensitive to this factor as a result of destruction of the VMH (49). Subsequent parabiotic experiments between mice with spontaneous mutations rendering them obese supported the existence of an appetite-suppressing factor related to adipose mass. Parabiosis of obese *ob/ob* mice and lean wild-type mice resulted in reduced feeding and weight loss in the *ob/ob* mice (50). Conversely, when wild-type or *ob/ob* mice were paired with genetically obese *db/db* mice, body weight was dramatically reduced in the wild-type and *ob/ob* mice (50, 51). It was postulated that the *ob/ob* mice could respond to a circulating satiety factor that was absent in its own circulation. In contrast, the *db/db* mice were proposed to produce this factor but were insensitive to its effects. Although these studies clearly indicated the probability of the existence of a circulating molecule that could signal satiety, it would be many years before the mysterious factor would be discovered.

1.5. Leptin

Decades following the earliest parabiotic experiments, the product of the *ob* gene, leptin, was discovered which met the criteria of the elusive circulating factor. The *ob* gene was identified in 1994 by use of positional cloning techniques, and encodes a 167 amino acid product that resembles members of the long chain helical cytokine family (52). Mouse and human *ob* genes are localized to chromosomes 6 and 7q31.3, respectively (53, 54). The 650 kilobase *ob* gene is comprised of 3 exons separated by 2 introns, in which the coding region for the Ob protein is located in exons 2 and 3 (52). Promoter analysis of the *ob* gene reveals cAMP and glucocorticoid response elements, CCATT/enhancer and SP-1 binding sites (55-57). There is a high degree of homology as the human prototype is 84% and 83% homologous to the mouse and rat proteins, respectively (52, 58).

One year following the discovery of leptin, a number of studies were published that observed the effects of leptin in wild-type, *ob/ob* and *db/db* mice (59-62). *Ob/ob* and *db/db* mice are characterized by obesity, hyperinsulinemia, hyperglycemia, and insulin resistance, a syndrome resembling diabetes and obesity in humans (63). Exogenous administration of recombinant leptin dramatically reduced body weight and normalized plasma glucose and insulin concentrations in the *ob/ob* mice (59-62). Whereas food restriction led to the loss of both lean body mass and adipose tissue, leptin-induced weight loss was specific for adipose tissue mass (61, 64, 65). While earlier studies attributed the weight loss to a reduction in appetite, studies in which control mice were pair-fed to the amount of food consumed by leptin-treated mice showed that leptin exerted weight-reducing effects in excess of those induced by caloric restriction alone (65). Increased energy expenditure may contribute to weight loss since leptin increased sympathetic outflow to brown adipose tissue and induced expression of mitochondrial upcoupling proteins (UCPs) (66-68). UCPs disrupt the mitochondrial proton gradient, resulting in the generation of heat rather than ATP. In comparison to pair-fed animals that displayed reduced levels of UCP mRNA in liver, muscle and brown and white adipose tissues, leptin treatment resulted in

high levels of UCP in all tissues (68), suggesting that leptin prevents the reduction in energy expenditure normally associated with a decreased food intake. Thus, leptin mediates its weight reducing effects through the combined effects of appetite suppression and induction of energy expenditure.

In contrast to its effects in the *ob/ob* mice, exogenous leptin treatment has no effects in the *db/db* mice (61, 69). Although both strains have similar obese and diabetic phenotypes, *db/db* mice are unresponsive to the actions of leptin. Following the identification of the leptin receptor (ObR) in 1995 (70), it was soon determined that *db/db* mice harbor a mutation in the *ObR* gene resulting in the production of a defective leptin receptor (71, 72). These findings are consistent with the earlier parabiotic studies in which it was postulated that *ob/ob* mice lack a circulating factor that regulates adiposity whereas *db/db* mice produce this factor but are resistant to its effects (50, 51). This mysterious factor is now known to be leptin.

Leptin is secreted as a 16 kilodalton protein chiefly from adipose tissue (52). In *ad libitum* fed animals, the levels of leptin mRNA and protein in adipose tissue and plasma are positively correlated to body fat and adipocyte size (73-75). Its expression has also been detected at low levels in placenta (76), stomach (77), muscle (78), brain (79, 80), and hepatic stellate cells (81). Leptin is synthesized and released in response to net energy surplus. One mechanism by which increased energy is sensed and translated to increased expression of the *ob* gene is through the hexosamine biosynthetic pathway (78). Although it accounts for only a small fraction of the glucose flux, glucose converted to fructose-6-phosphate can enter the hexosamine biosynthetic pathway in which the end product, UDP-GlcNAc is utilized in glycosylation of proteins. Induction of this hexosamine biosynthetic pathway stimulates *ob* gene expression in muscle and fat, providing a link between increased availability of nutrients and leptin expression (78).

Leptin produced by adipose tissue relays information about adipocyte metabolism and stores to appetite centers in the hypothalamic regions of the brain (82, 83). This signal forms the afferent pathway of a distinctive feedback loop

that regulates feeding behavior. Plasma leptin levels correlate closely with the percentage of body fat so as adipose stores rise, leptin is produced and secreted in greater quantities. Evidently, adipocyte size is as important as adipocyte numbers in determining leptin secretion, as larger adipocytes produce more leptin than smaller adipocytes in the same individual (84). Alternatively, as body weight falls and fat mass decreases, the reduction in leptin levels is detected by the CNS, which in turn decreases energy expenditure and reduces satiety in an attempt to increase nutrient storage and body weight. The action of leptin is part of a long-term system that balances food intake and energy expenditure and regulation of the body's energy stores. Unlike factors such as plasma glucose concentration, body temperature, plasma amino acids and cholecystokinin that regulate meal patterns and feeding behavior throughout the day (85), leptin levels do not increase after a meal nor lead to termination of a meal in humans (86). In contrast to rodents in which leptin levels are transiently increased after feeding (87), significant increases in leptin concentrations in humans have generally been observed after several days of overfeeding or refeeding following a period of starvation (88, 89). In both rodents and humans, however, decreases in leptin expression in response to fasting occur within several hours after initiation of fasting and disproportionately to corresponding changes in body weight or body fat (87, 89-92). A decrease in leptin levels may therefore serve a more critical role than a rise in leptin concentrations in signalling nutritional status. The rapid fall in leptin levels may be an important signal during periods of food deprivation that allows for the body to adapt by conserving energy and increasing food-seeking behavior (93). Given the evolutionary benefit of efficiently protecting fuel stores during periods of food deprivation, it would appear probable that the purpose of reducing leptin is to assure that sufficient caloric reserves are maintained.

Leptin expression is determined in part through nutritionally regulated hormones. Leptin production by white adipose tissue is stimulated by insulin and cortisol and attenuated by β-adrenergic agonists, cAMP and thiazolidinediones.

Leptin levels are reduced in states of low insulin, like fasting and streptozotocin-induced diabetes (92, 94) in which pancreatic islets are targeted and destroyed. Conversely, maintenance of hyperinsulinemic states by insulin infusion results in a correlative rise in leptin expression (95-97). Elevated levels of glucocorticoids results in development of a Cushing's-type obesity characterized by central deposition of fat. An associated increase in insulin levels ensues which may partly explain glucocorticoid-induced hyperleptinemia. However, glucocorticoids can directly stimulate leptin synthesis in cultured adipocytes (98, 99) and induction of *ob* gene expression by corticosteroids is accompanied by weight loss and reduced food intake (100). Elevated levels of corticosteroids and insulin are both suspected of contributing to leptin-induced auto-downregulation of its own signal (101, 102).

In terms of the relationship between leptin and thyroid hormones, a number of conflicting reports have been produced and there is currently insufficient data to definitively correlate leptin expression in either hypothyroid or hyperthyroid states (103-107). Leptin, in turn, increases triiodothyronine and decreases thyroxine, increasing the rate of conversion of thyroxine to triiodothyronine and altering thyroid activity (108, 109). Therefore, a relationship appears to exist between leptin and thyroid hormone although the effect of thyroid hormone on leptin expression remains to be clarified.

1.6. Leptin Receptor

Using the technique of expression cloning of complementary DNA taken from the choroid plexus of mice, the first leptin receptor (ObR) was isolated in 1995 shortly after the discovery of the *ob* gene (70). The leptin receptor family is structurally related to the class I cytokine receptor family, with a similar secondary structure (70). Multiple splice variants of ObR mRNA encode at least 6 leptin receptor isoforms (ObRa, ObRb, ObRc, ObRd, ObRe and ObRf) (110, 111), which have similar extracellular domains but differ mainly by their intracellular domains (71). The long receptor isoform, ObRb is predominantly

expressed in the VMH region known to be important in determining feeding behavior, and it is now recognized that ObRb is expressed in a wide array of peripheral tissues (83, 112-114). While ObRa, ObRb, ObRc, ObRd and ObRf all contain intracellular motifs, only the long intracellular domain of ObRb of 304 amino acids contains putative motifs for binding of Janus protein tyrosine kinase (JAK) and signal transducers and activators of transcription (STAT). JAK and STAT binding are key steps for cytokine class I receptor signalling. Leptin binds to a homodimer of ObRb in a 1:1 (ligand:receptor) ratio (115, 116), which leads to phosphorylation of JAK2 (Fig. 1.1). Upon activation of JAK2, the intracellular domain of the receptor becomes phosphorylated which then allows for binding and activation of the STAT proteins. Dimerization of the STAT proteins causes their translocation to the nucleus, where they regulate gene transcription (117). In addition to the JAK/STAT pathway, signal transduction via ligand binding to the ObRb receptor may involve the mitogen-activated protein kinase (MAPK) (118) and phosphatidylinositol-3-kinase (PI3K) pathways (119).

ObRb appears to be involved in relaying the physiological actions of leptin. The *db/db* mouse and *fa/fa* rat, which have a phenotype similar to the *ob/ob* mouse, do not express functional ObRb, suggesting that this isoform is crucial for leptin signalling (71, 72). The short forms of the leptin receptor are widely expressed throughout the body and are presumed to signal via the JAK and MAPK pathways, albeit substantially weaker than ObRb (118). The role of the short receptor isoforms has yet to be clearly defined, although it has been suggested that they play a role in mediating the uptake of leptin into the CNS as well as regulating its turnover. The expression of the ObRa in the choroid plexus has implicated this short receptor isoform as a player in the movement of leptin from the circulation across the blood-brain barrier. A saturable transport mechanism has been shown to be the means of leptin translocation and in this regard, ObR may be a critical factor (120). Of the short forms of the receptors, ObRe lacks transmembrane and intracellular domains and circulates as a soluble receptor (71). ObRe receptor binds leptin in the blood and modulates steady-state

leptin levels by complexing free leptin in the circulation and preventing its degradation and clearance (121). Leptin circulates in both free and bound forms (122). In lean individuals, the majority of leptin circulates in the bound form whereas in obesity, decreased amounts of bound leptin and ObRe are observed (122-125). Therefore, soluble leptin receptor may be a critical factor in determining the amount of circulating bioactive leptin that is perceived by the body.

Hypothalamic nuclei that are implicated in the control of energy homeostasis include the ventral medial hypothalamus (VMH), which is involved in the regulation of satiety and the lateral hypothalamus (LH), an area involved in the hunger state. Insulin, which also acts in the brain to reduce energy intake (126), shares similar pathways (for a comprehensive review, see (127). The suppression of food intake/weight gain is influenced by neurons/fibre tracts in the VMH/DMH, an area also involved with increasing energy expenditure, while a reduction of energy usage and the stimulation of food intake is influenced by neurons in the LH (128). These hypothalamic centres receive signals from the arcuate nucleus, a collection of neuronal cells bodies situated adjacent to the floor of the third ventricle. Various cell populations are housed in the nucleus including neuropeptide Y/agouti-related peptide (NPY/AGRP) and proopiomelanocortin/cocaine- and amphetamine-regulated transcript (POMC/CART) neurons (129-131). Leptin inhibits NPY/AGRP neurons, preventing the secretion of NYP and AGRP, neuropeptides that stimulate food intake (62, 132). Conversely, leptin stimulates POMC/CART neurons (133), eliciting the release of α-melanocyte-stimulating hormone, which acts on its melanocortin receptor to suppress feeding. The reduction in leptin during fasting stimulates NPY/AGRP (62, 129, 130, 132) and suppresses CART/POMC (134-136) neurons. Through these hypothalamic centers, leptin communicates the current status of nutrition to the brain, and thereby induces appropriate compensatory responses during feeding and fasting.(128)(137).

1.7. Other Actions of Leptin

While body weight regulation is established as an important role for leptin, leptin has a wide range of other effects on physiological processes, including the neuroendocrine, reproductive and immune systems and regulation of hematopoiesis, bone and brain development (138-141). Many of these processes are dependent on adequate nutritional intake. Leptin receptors are expressed on immune cells and leptin is capable of stimulating an immune response by directly regulating lymphocyte proliferation and differentiation (142). Leptin also activates gonadal and thyroid endocrine axes and suppresses the adrenal axis. Leptin declines rapidly during fasting and triggers a rise in glucocorticoids and reduction in thyroxine, sex and growth hormones (93, 137). The absence of leptin is associated with hypothalamic hypogonadism in humans and rodents (143). Leptin treatment restores LH secretion and pubertal development in leptin-deficient patients (144) and it appears that leptin evolved as a means of assuring that sexual maturation and pregnancy would not occur in the absence of sufficient fuel stores for fetal growth and suckling. A fall in leptin also results in suppression of the immune system during starvation (145). Therefore it would appear that reduced leptin levels promote energy intake and limits the diversion of energy towards such processes as reproduction and immune response.

1.8. Clinical Uses of Leptin

The clinical phenotype of human congenital leptin deficiency is very similar to that seen in the *ob/ob* mice (143, 144, 146, 147). Similar to the *ob/ob* mouse, leptin-deficient humans have voracious appetites, early-onset obesity, impairment in sexual development, deregulation of the hypothalamo-pituitary thyroidal axis and defects in immune mediated responses (143, 144, 146, 147). The key differences between rodents and humans are elevated corticosteroid levels and reduced energy expenditure in the *ob/ob* mice but not in humans (144, 146, 148, 149). As a result, the major effect of leptin treatment in three children with leptin deficiency, due to a frameshift mutation (ΔG133) in the *ob* gene, was

normalization of hyperphagia as there were no significant effects on basal metabolic rate or energy expenditure (144, 147). Leptin treatment reduced their circulating insulin levels as well as the serum concentrations of cholesterol and triglycerides. Other parameters were also improved by leptin therapy including increased thyroid hormone production, restoration of pubertal development and improved immune function. Although all three children with the ΔG133 mutation had normal fasting plasma glucose values, one individual of Turkish descent was found to have a different missense mutation resulting in leptin deficiency and was hyperglycemic (143). The mutation described in this individual and 2 additional members from her family is identical to the mutation that leads to the presence of a premature stop codon in the *ob/ob* mouse (52). Although recombinant leptin therapy has not yet been studied in this family of congenital leptin deficiency, it is likely that leptin may reduce plasma glucose concentrations in these individuals similarly to its effects in *ob/ob* mice. These findings provide evidence for the therapeutic use of leptin in treating the multiple phenotypic abnormalities associated with congenital leptin deficiency.

Lipodystrophy, caused by a deficiency or destruction of adipose cells, is another state characterized by low leptin levels (150). Although these individuals are not obese, major phenotypic manifestations include insulin resistance, hyperglycemia, hyperinsulinemia, hypertriglyceridemia, and hepatic steatosis. Leptin therapy ameliorates these features, increasing insulin sensitivity, reducing plasma circulating levels of insulin, glucose and triglycerides, and reducing lipid content in non-adipose tissues (151-154). These studies have provided valuable insight into the effects of leptin that are independent of satiety and weight loss.

1.9. Role of Leptin in Lipid Homeostasis

Leptin levels of greater than 10 ng/ml such as that which circulates in obese mice evidently exceeds the threshold determined by the blood-brain barrier (155). Therefore, the role of hyperleptinemia may be to confine the storage of triglycerides to adipocytes while limiting triglyceride storage in non-adipocytes,

thus protecting them from lipotoxicity (156, 157). The extra-adipocyte accumulation of triglyceride is known as steatosis, a condition that affects liver, skeletal muscle, myocardium and pancreatic β cells, resulting in impaired insulin signalling and secretion and myocardial function (158). Studies of animal models of lipodystrophy that received leptin administration (159) or fat tissue implants from normal but not from *ob/ob* mice (160) clearly demonstrated the anti-steatotic effect of leptin. The anti-diabetic effects of leptin in these animals appeared to come from leptin's ability to stimulate lipolysis and fatty acid oxidation in liver and skeletal muscle, slowing the progression of steatosis by preventing accumulation of toxic metabolites such as ceramide (159). Tissue-specific overexpression of leptin receptors in steatotic livers of *fa/fa* rats reduced the accumulation of triglycerides (156) and adenovirus-mediated hyperleptinemia in rats increased intracellular oxidation of fatty acids resulting in the depletion of triglyceride content in liver, muscle and pancreas (157). Therefore, leptin appears to preferentially up-regulate fatty acid oxidation and reduce triglyceride accumulation in non-adipose tissues.

A reduction in triglyceride content in non-adipose tissue, such as skeletal muscle, following chronic administration of leptin may be accounted for in part, through a reduction of fatty acid uptake or increased fatty acid oxidation and triglyceride hydrolysis (157, 161-163). Leptin reduced the expression of fatty acid transporter protein and fatty acid binding protein, and thereby reduced fatty acid transport across the sarcolemma in skeletal muscle (161). Increased rates of fatty acid oxidation may be explained by increased activation of the enzymes involved in lipolysis, including hormone-sensitive lipase and carnitinepalmitoyltransferase 1 in skeletal muscle (164), and malic enzyme and lipoprotein lipase in liver and adipose tissue (165, 166). Leptin inhibited lipogenesis by suppressing the activities of glycerol phosphate dehydrogenase and acetyl-CoA carboxylase, enzymes involved in the biosynthesis of long chain fatty acids, in a preadipocyte cell line (167). Acetyl-CoA carboxylase, the rate-limiting enzyme of fatty acid synthesis, directs fatty acids towards triglyceride

biosynthesis and away from oxidation. 5'-AMP-activated protein kinase (AMPK) phosphorylates and inhibits acetyl-CoA carboxylase activity resulting in a subsequent increase in fatty acid oxidation. Leptin stimulated fatty acid oxidation in muscle through both a centrally mediated and direct effect by activating AMPK (168, 169). The lipogenic transcription factor, sterol regulatory element binding protein 1 was up-regulated in liver and islets of *fa/fa* rat and thus may also play a role in the lipogenesis and lipotoxicity complicating obesity in diabetic fatty rats (170). Furthermore, leptin repressed RNA levels and enzymatic activity of hepatic stearoyl-CoA desaturase-1, which catalyzes the biosynthesis of monounsaturated fatty acids (171). Through an increase in lipolysis and reduction in fatty acid uptake and triglyceride synthesis, leptin up-regulates fatty acid oxidation in all tissues and prevents ectopic lipid over-accumulation in non-adipocytes, thus sparing them from the metabolic consequences of lipid overload with obesity (158).

1.10. Role of Leptin in Glucose Homeostasis

It was originally believed that the effects of leptin on glucose metabolism are attributed to weight loss, which in itself can affect glucose homeostasis. In one of the earliest studies, Pelleymounter *et al.* demonstrated that leptin treatment of *ob/ob* mice at doses that did not prevent body weight gain, normalized the hyperglycemia and hyperinsulinemia in these mice (59), supporting a role of leptin in regulating glucose homeostasis independently of its effects on body weight. In studies where a cohort of *ob/ob* mice were pair-fed to consume the same amount of food as mice treated with leptin, pair-fed mice experienced reductions in glucose and insulin levels that were <60% of the reduction induced by leptin (132). Thus, leptin had effects on both glucose and insulin concentrations that were beyond those of caloric restriction alone. In rats administered leptin by osmotic mini-pumps for 8 days, an enhancement in the actions of insulin on stimulating glucose uptake and inhibition of hepatic glucose production were greater than in calorie restricted, pair-fed mice (172). In

addition, subcutaneous infusion of leptin for 48 h in lean rats resulted in reduced serum glucose concentrations and improved glucose utilization both under fasting conditions and during a hyperinsulinemic glucose clamp (173). The acute effect of leptin was demonstrated by a single intraperitoneal injection of leptin in *ob/ob* mice, which resulted in dramatic reductions in plasma insulin and glucose after 24 h, when no changes in body weight were observed (174). Furthermore, both intracerebroventricular (ICV) and intravenous (IV) infusion of leptin for 5 h into wild-type mice increased glucose turnover without altering plasma insulin concentrations (108). These observations suggest that leptin can regulate glucose homeostasis and that these effects are independent of weight loss or reductions in food intake.

The effects of leptin on glucose metabolism could be in part mediated by the CNS. Microinjection of leptin in the ventromedial hypothalamus but not lateral hypothalamus increased glucose uptake in brown adipose tissue, heart, skeletal muscle and spleen but not white adipose tissue or skin (175). Further supporting a role for central regulation in leptin's actions on peripheral glucose utilization, surgical denervation of brown adipose tissue prevented the effects of leptin on glucose uptake in this tissue (176). Sympathetic innervation appeared to be important in mediating the effects of leptin as adrenergic antagonists such as guanethidine inhibited leptin-stimulated glucose uptake in heart, brown adipose tissue and skeletal muscle in lean rats (176). In this study, a synergistic effect of leptin on insulin mediated glucose uptake was also demonstrated (176). In rats made hyperglycemic by streptozotozin destruction of pancreatic β cells, ICV leptin improved insulin sensitivity, normalizing glucose concentrations independently of food intake and changes in insulin levels (177-179). Improvements in insulin sensitive pathways in the absence of insulin suggested that leptin mimics insulin action, possibly through such intermediates as PI3K and MAPK (180). These studies demonstrated that whole body glucose homeostasis may be regulated, in part, by leptin through CNS-regulated pathways.

Although it is commonly regarded that the central pathways are essential for integrating the leptin signal and relaying its physiological effects, there arises increasingly more evidence to substantiate the hypothesis that leptin may, in fact, also act directly on peripheral tissues. The introduction of a fully functional *ObRb* gene into the brains of *db/db* mice that normally produce a defective ObRb receptor failed to completely correct the obesity phenotype (181). Neuron specific expression of ObRb corrected the thermogenic defects in *db/db* mice but did not fully restore food consumption to levels in lean control mice. In addition, *db/db* mice with neuron specific expression of ObRb maintained higher plasma concentrations of both glucose and insulin in comparison to lean mice. These findings may be attributed to several factors such as uneven distribution of the *ObRb* gene resulting in its absence in some hypothalamic neurons or the failure for the gene to produce appropriate numbers of leptin receptors in certain neurons. Another possibility is that *ObRb* gene expression is required in peripheral tissues for comprehensive leptin action on feeding behavior and glucose homeostasis. Leptin receptors are widely distributed in peripheral organs (83, 112-114) supporting the notion that leptin mediates actions that are independent of those of the CNS. Regulation of glucose metabolism requires integration of actions in pancreatic β cells, muscle adipose tissue and liver.

1.10.1. *Leptin and Insulin Secretion*

In the absence of leptin or functional leptin receptors as in the *ob/ob* and *db/db* mice, respectively, hyperinsulinemia manifests as one of the first metabolic abnormalities, preceding the development of insulin resistance, feeding abnormalities or weight gain (182-190). It would make sense then that the normal response of the pancreatic β cells to leptin is to reduce insulin secretion. Treatment of *ob/ob* mice ameliorated hyperinsulinemia (59, 62, 65, 132, 191, 192) and this may partly be attributed to a direct reduction of insulin secretion by leptin from β cells since leptin receptors have been detected in β cells (193). The acute reduction in plasma insulin levels after leptin treatment did not appear to be

secondary to either diminished body weight or a fall in plasma glucose levels in hyperglycemic rodents. Despite some earlier studies that demonstrated a lack of a leptin effect on the pancreas or an enhancement of insulin secretion by leptin, the current data generated from experiments in perfused rat pancreas, and isolated rat, mouse and human islets, is increasingly supportive of a role for leptin as an inhibitor of insulin release (reviewed in (194)). In support of a direct effect of leptin on β cells, leptin has been reported to inhibit insulin secretion from isolated islets and tumour-derived β cell lines. Static incubation or perifusion of leptin reduced insulin secretion either in the presence of basal or high levels of plasma glucose concentrations in isolated islets from mice and rats, and from the perfused rat pancreas (193, 195-201). Similar results have been attained from studies on cultured human islets (202, 203) and in various tumour derived β cell lines (195, 197). These findings provide evidence in support of a direct effect of leptin on insulin secreting pancreatic β cells.

Leptin acts to suppress the release of insulin by glucose and other insulin secretagogues. Glucose is a potent stimulant of insulin secretion from β cells although the precise biochemical cues which couple the entry of glucose into β cells to insulin release have yet to be fully elucidated. It is generally accepted that the increase in ATP production resulting from the intracellular metabolism of glucose is responsible for triggering the start of a complex cascade of events that ultimately leads to insulin secretion. The increase in ATP has been shown to promote the closure of ATP-sensitive K^+-channels (K_{ATP}), which results in membrane depolarization and activation of voltage-dependent L-type Ca^{2+} channels (VDCC) (204). The opening of VDCC leads to influx of Ca^{2+} down its concentration gradient. The Ca^{2+} binds to calmodulin and subsequently activates Ca^{2+}/calmodulin-dependent protein kinase (CaMK). CaMK is thought to be associated with cytoskeletal structures and may be involved in the regulation of transport and fusion of insulin-containing vesicles to the plasma membrane (205).

It is important to appreciate that the aforementioned events do not sufficiently account for the physiological regulation of insulin secretion in β cells.

Indeed, glucose-induced insulin secretion can be affected by a mechanism distinct from the closure of K_{ATP} channels and increases in the concentration of intracellular Ca^{2+} ($[Ca^{2+}]_i$) (206, 207). Metabolic factors other than ATP have also been shown to be coupled to glucose-induced insulin secretion. These include long chain fatty acid CoA (LC-CoA) and malonyl-CoA. Following uptake, glucose is metabolized into malonyl-CoA in the β cell (208). Malonyl-CoA inhibits carnitine-palmitoyl transferase I, the rate limiting enzyme in mitochondrial fatty acid oxidation, thereby facilitating the accumulation of LC-CoA (209). It is hypothesized that LC-CoA is required for the budding of insulin-containing vesicles from cis-Golgi, inducing the fusion of secretory granules with the plasma membrane (210). LC-CoA may also play a role in regulating the expression levels of genes encoding metabolic enzymes involved in fuel sensing in the β cell (211, 212). However, several lines of evidence contradict the role of malonyl-CoA and LC-CoA in facilitating insulin secretion (213-215). Overexpression of malonyl-CoA decarboxylase, an enzyme that decarboxylates malonyl-CoA to acetyl-CoA, resulting in low levels of malonyl-CoA, failed to inhibit glucose-stimulated insulin secretion (216). Similarly, triacsin C, a pharmacological agent that blocks free fatty acid conversion to LC-CoA, had no effect on insulin secretion from β cell (216). Therefore, although there may be a role of malonyl-CoA and LC-CoA in stimulating insulin secretion, the relevance of this pathway remains controversial.

The observation that islets from the leptin resistant *db/db* mice are partially depolarized (217) suggests that leptin may normally hyperpolarize β cells and thereby reduce cytosolic free Ca^{2+} and insulin exocytosis (218). Whole cell current clamp recordings demonstrated that in isolated β cells from *ob/ob* mice (218) and the insulin secreting cell line CRI-G1 (219, 220), leptin treatment caused hyperpolarization. Leptin also increased membrane conductance in *ob/ob* mouse β cells. The increase in conductance was reversed by the application of the sulfonylureas, tolbutamide and glibenclamide, which specifically inhibit K_{ATP} channels (219-221). Furthermore, measurements of single channel activity in

ob/ob mouse β cells and CRI-GI cells showed that leptin increased K_{ATP} open channel probability with no significant effect on open times (218, 219), indicating that leptin acts upon K_{ATP} channels. Subsequent downstream reductions in $[Ca^{2+}]_i$ could explain the suppressive effects of leptin on insulin secretion. Leptin attenuates $[Ca^{2+}]_i$ elevations induced by glucose in isolated mouse and human β cells (202, 218, 222), and in several β cell lines (195, 197). Leptin may also potentially suppress insulin release by promoting triglyceride depletion through increased β-oxidation to free fatty acids (157, 223). The metabolically active form of free fatty acids is the fatty acyl CoA esters, which bind to and open K_{ATP} channels (224, 225). Thus, leptin potentially targets the products of lipid metabolism to activate K_{ATP} channels.

In addition to inhibiting insulin secretion, leptin reduces insulin biosynthesis by regulating pro-insulin gene expression. Steady state levels of preproinsulin mRNA were reduced after a single leptin injection compared to vehicle treated animals (174). Similar results were obtained *in vitro* when isolated *ob/ob* islets were incubated with leptin for 24 h (174). Suppression of preproinsulin mRNA by leptin was also observed in islets isolated from rats and humans and in tumour-derived β cell lines (174, 195, 198, 222). The inhibitory effects of leptin on gene expression appeared to be independent from the activation of K_{ATP} channels. Leptin was capable of reducing the insulin gene promoter activity in INS-1 cells in both the presence and absence of diazoxide (174). Diazoxide, which, like leptin, opens K_{ATP} channels, had no acute effect on insulin gene expression nor altered the ability of leptin to reduce insulin gene expression (174). This observation suggested that the effects of leptin on insulin production may be mediated through different intracellular signalling pathways. A potential pathway by which leptin relays its effects on insulin biosynthesis in the β cell is through STAT proteins. Several STAT proteins have been implicated in leptin action in pancreatic islets and cell lines, including STAT1, 3, and 5 (174, 226, 227). In addition, it has been speculated that leptin's suppression of insulin gene transcription is mediated in part, through the MAPK pathway (226, 227).

Given that insulin stimulates leptin production, there appears to be a bidirectional feedback loop between adipose tissue and the endocrine pancreas, which has been referred to as the adipoinsular axis (193). Leptin serves as one arm of this bidirectional feedback system that allows for communication between adipose tissue and β cells. Completing the loop is insulin, which is known to have an effect on increasing leptin gene expression and levels in white adipose tissue (96, 228-234). An adipoinsular action may be important in the regulation of fat deposition and glucose homeostasis. Insulin stimulates leptin production from fat tissue. As fat mass accumulates and further energy storage is no longer required, leptin would suppress production and secretion of insulin, a major adipogenic hormone. This feedback system allows for physiological control of insulin secretion to needs determined by body fat stores. Defects in insulin secretion as a result of deregulation of the adipoinsular axis is one theory that may explain early signs of hyperinsulinemia that is frequently observed to precede insulin resistance and the development of diabetes.

1.10.2. *Leptin Effects on the Gastrointestinal System*

The discovery that the stomach is a source of leptin prompted investigations into the role that leptin plays in regulating nutrient absorption (77). The presence of leptin receptors in human gastric mucosa suggests a paracrine and/or autocrine effect of leptin on gastric epithelial cell function (235, 236). Although gastric leptin has been found to be secreted in response to feeding, administration of a biologically active cholecystokinin derivative (77), and vagal stimulation (237), a role for gastric leptin in the induction of satiety has not yet been clarified. However, duodenal infusion of leptin in rats has been shown to increase plasma CCK levels, and thereby induce satiety (238).

Although it remains unclear as to whether leptin mediates effects on receptors in the stomach, leptin has been reported to enhance small intestine carbohydrate absorption. ObRb is distributed throughout the cytoplasm of the enterocytes, of both villi and crypts, and in the basolateral plasma membrane

(239-241). Although a distinction has not been made with regards to the source of leptin that acts in the small intestine, leptin has been shown to inhibit D-galactose absorption in rat small intestinal rings (242) potentially through a protein kinase A dependent mechanism (243). Activation of STAT3 and STAT5 by leptin has also been demonstrated in a human model of small intestine epithelium, CACO-2 cells and STAT signalling in response to leptin has been demonstrated in jejunum and weakly in ileum (239). Leptin might also play a physiological role in lipid handling by reducing the apolipoprotein AIV transcript (Apo-AIV) and thereby reducing the concentrations of secreted triglycerides (239, 244). The Apo-AIV system serves to transport triglycerides as chylomicrons into the circulation. Other lipid profiles altered by leptin include the reduction of ApoB100 and ApoB48 and output of chylomicrons and low density lipoproteins (240). Further studies are needed to clarify the role of leptin production and leptin receptors in the gastrointestinal tract.

1.10.3. *Leptin Effects on Skeletal Muscle*

Leptin regulation of glucose utilization by muscle may contribute to the reduction in plasma glucose observed upon leptin administration to *ob/ob* mice since skeletal muscle accounts for a large proportion of insulin-stimulated glucose disposal from the blood. *In vivo*, IV or ICV administration of leptin for five hours in wild-type mice increased glucose uptake in skeletal muscle and whole-body glycolysis (108). These observations were made in the absence of changes in blood insulin concentrations, implying that insulin sensitivity was improved in skeletal muscle. These effects were initially attributed to central leptin action since leptin was unable to stimulate glucose uptake by extensor digitorum longus (EDL) muscle when the leg was denervated. Interestingly, glucose uptake in denervated soleus muscle was slightly increased in response to leptin. These results may be indicative of a direct action of leptin on muscle receptors. Although several studies support this finding of an increase in glucose utilization by muscle by leptin *in vivo* (175, 245-249), other reports have demonstrated no

role or a minor role of skeletal muscle in the acute disposal of glucose following leptin treatment (250-252). Based on these studies, the extent to which leptin regulates glucose utilization through direct actions in skeletal muscle remains unclear.

Due to these contradictory findings *in vivo*, it has been of considerable interest to determine whether leptin is able to affect glucose metabolism in skeletal muscle *in vitro*. The first evidence of a direct effect of leptin is the presence of the long form of the leptin receptor in skeletal muscle (70, 112, 253). While some studies have demonstrated an effect of leptin on basal and insulin-stimulated glucose uptake in muscle isolated from lean and *ob/ob* mice or cultured myocytes (254-258), other studies have observed no direct effect of leptin (259-261). In one study, preincubation of L6 myotubes with leptin had no effect on basal glucose uptake but reduced insulin-stimulated glucose uptake by suppression of MAPK phosphorylation (262). Leptin has also been reported to have no effect (261) or an inhibitory effect on glycogen synthesis (263). Conflicting with these findings, in isolated mouse soleus muscles from lean rats and a skeletal muscle cell culture, C_2C_{12} myotubes, leptin stimulated glucose uptake and lactate formation and potentiated the effect of insulin on glucose incorporation into glycogen (254-256). Increased decarboxylation of glucose by leptin in isolated soleus muscle demonstrated insulin-mimetic effects of leptin in also promoting glycolysis (257). In a study by Ceddia *et al.* (257), leptin enhanced the effects of insulin on glucose decarboxylation but independently stimulated glucose transport and glycogen synthesis, implying that leptin may relay its signal via intermediates of the insulin signalling pathway or that crosstalk between the leptin and insulin signalling pathways may exist. In C_2C_{12} myotubes, leptin activated glucose transport and glycogen synthesis through a mechanism that involved the PI3K pathway (255) and extracellular signal-regulated kinase 2 (ERK2) activity (264). Enhanced levels of glucose uptake may be attributed to recruitment of GLUT4 to the cell surface, which facilitates an increase in glucose flux into the cell. The mechanism by which PI3K is activated upon leptin

stimulation may involve JAK2 phosphorylation of IRS proteins (265, 266), in particular IRS-2 (265). Alternatively, the effect of leptin on glucose uptake in muscle may be attributed in part to bradykinin and nitric oxide (258), two other factors that have previously been demonstrated to be involved in exercise mediated glucose uptake (267-269). Regarding the direct effects of leptin in skeletal muscle, the variability in these reports to date may be attributed to different cell lines and culture conditions. Taking these results into consideration, a direct action of leptin on skeletal muscle remains debatable and further studies are warranted to clarify the role of leptin in this tissue.

1.10.4. *Leptin Effects on Adipose Tissue*

The presence of leptin receptors in adipose tissue (112, 165) suggests that leptin may also have direct actions in this tissue. Leptin signalling through phosphorylation of JAK and STAT proteins is present in adipocytes (165, 270) and several *in vitro* studies have demonstrated a direct effect of leptin in adipose tissue (165, 270-273) although other studies have also reported no effect of leptin on glucose metabolism in adipocytes (259, 260, 274, 275). It is therefore, of interest to clarify whether adipose tissue may be another peripheral tissue upon which leptin acts to regulate glucose metabolism.

While the direct effects of leptin on glucose metabolism in skeletal muscle remains uncertain, leptin has consistently been shown to increase glucose uptake and utilization in brown adipose tissue. Leptin had an insulin sensitizing effect when administered ICV, stimulating glucose uptake and utilization in brown adipose tissue in lean mice and rats (68, 108), presumably through a mechanism that involved an increase in GLUT4 mRNA (246). Surgical sympathetic denervation of brown adipose tissue abolished the enhancement of glucose uptake by leptin delivered into the VMH (175, 176), thus suggesting that the effects of leptin were mediated centrally. However, intravenous administration of leptin in lean rats resulted in an increase in glucose utilization in brown adipose tissue that was not seen in brown adipose tissue from animals that were pair fed (165).

Therefore, these studies demonstrate that leptin has an insulin-like effect in increasing glucose utilization in brown adipose tissue.

In contrast to an insulin-mimetic effect in brown adipose tissue, leptin mediates an anti-insulin action in white adipose tissue. Leptin infusion for four days to lean rats resulted in a reduction in glucose uptake in white adipose tissue (276). *In vitro*, leptin inhibited glucose transport, glycogen and protein synthesis and lipolysis in isolated rat adipocytes (271, 277). Inhibition of glucose transport in white adipose tissue may be attributed to a reduction in GLUT4 mRNA (246). Furthermore, leptin inhibited the pyruvate dehydrogenase complex (PDH), the rate-determining step for glucose oxidation in white adipose tissue (166). These effects may be mediated by direct interference with insulin signalling as leptin was found to impair insulin-stimulated phosphorylation of the insulin receptor, PI3K and glycogen synthase kinase 3β, and MAPK activity in rat and mouse adipocytes (270, 278). An associated increase in suppressor of cytokine signalling-3 (SOCS-3) protein suggested that leptin may inhibit insulin action through SOCS-3 activation (278). Inhibition of insulin-stimulated glucose oxidation may be a defensive mechanism designed to prevent incorporation of glucose into lipids in white adipose tissue (272). This would be consistent with the role of leptin in promoting fatty acid oxidation. Collectively, these studies demonstrate that leptin suppresses glucose uptake and oxidation in white adipose tissue, actions which may facilitate the mobilization of fat and reduction of energy storage. Henceforth in this thesis, "adipose tissue" will refer to white adipose tissue.

1.10.5. *Leptin Effects on the Liver*

In *ob/ob* mice treated with leptin, fasting plasma glucose concentrations were dramatically reduced (59). In individuals with diabetes, a defect in liver metabolism resulting in increased gluconeogenesis is largely responsible for the enhanced hepatic glucose production and fasting hyperglycemia (16). Therefore, regulation of glucose metabolism in the liver may be one mechanism by which

leptin normalizes glycemia. Leptin administration in rats during a euglycemic hyperinsulinemic clamp augmented the inhibition of hepatic glucose production by insulin (172, 250). In the presence of leptin, adrenaline- and glucagon-stimulated glucose release was suppressed in perfused rat livers (279, 280). Furthermore, leptin infusion of streptozotocin-induced diabetic rats normalized the hyperglycemia and this was accompanied by an increase in mRNA expression for the glucose transporter, GLUT2, in the liver, presumably to facilitate an increase in glucose uptake into hepatocytes (179). Conversely, an increase in hepatic glucose production was observed in *ob/ob* mice receiving a continuous IV infusion of leptin (252) and presumably in wild-type mice that displayed reduced hepatic glycogen stores with ICV- and IV-infused leptin (108). The explanation for these discrepancies is currently not known. However, the inability to suppress hepatic glucose production is also characteristic of *fa/fa* rats that produce a defective leptin receptor (281), suggesting that leptin signalling is required for appropriate regulation of glucose flux in the liver. Therefore, it is proposed that leptin regulates glucose metabolism in the liver by reducing hepatic glucose production.

Detectable levels of ObRb mRNA are present in the liver, providing support for leptin activity in this organ (112, 196). Leptin has direct effects, *in vitro*, in hepatoma cells (119, 282-285), isolated hepatocytes (280, 286-290) and perfused rat livers (279, 280, 291, 292). These effects of leptin are primarily relayed through the JAK-STAT signalling pathway (180, 282, 293) although a cross-talk network established between leptin and insulin facilitates the interaction of leptin with intermediates of the insulin signalling cascade (288, 294). Originally, leptin was shown to inhibit insulin receptor substrate-1 (IRS-1) phosphorylation and Grb-2 association while increasing association of PI3K with IRS-1 in a human hepatocellular carcinoma HepG2 and rat hepatoma H4-II-E cell lines (119). In HepG2 and H4-II-E cells, ObRb was not detected and therefore, the effects of leptin were proposed to be due to signalling via short forms of the leptin receptor (119). In contrast, leptin enhanced insulin-induced IRS-1 and -2

phosphorylation in H-35 cells that were transiently transfected with the *ObRb* gene (282). In these cells, leptin was able to increase the association of PI3K with IRS-2 and this action was attributed to the presence of the long form of the leptin receptor (282). Leptin was also reported to have opposing actions on insulin-stimulated IRS-1 and -2 tyrosine phosphorylation within the same cell, in ObRb-expressing FAO hepatoma cells (283). Insulin-induced association of the p85 subunit of PI3K with IRS-1 was increased whereas that with IRS-2 was reduced. The physiological relevance of the divergence in these signalling pathways is not yet clear (283). However, these studies provide evidence that leptin mediates direct actions in the liver.

While these findings suggest that leptin modifies insulin action in the liver, leptin may also independently activate insulin signalling. In isolated rat hepatocytes, both insulin and leptin stimulated the association of the p85 subunit of PI3K with IRS-1 and IRS-2 (288). These observations were attributed to leptin signalling tthrough the short forms of the leptin receptor via JAK2 activation, which has previously been implicated as a mechanism by which leptin stimulates phosphorylation of IRS-1 (118). One pathway that has been assigned to the short forms of the leptin receptor is the activation of PI3K via IRS-1 and -2, and downstream molecules, protein kinase B and phosphodiesterase 3B (288). Interestingly, this theory is supported by the *in vivo* IV administration of leptin to lean rats which acutely increased IRS-2- and p85-associated PI3K activity and phosphorylation of MAPK and PI3K with minimal activation of STATs in the liver (180). These observations suggest that leptin may signal via various signalling pathways to elicit its metabolic effects, both independently and dependently of insulin signalling.

One mechanism by which leptin can reduce plasma glucose concentrations is by increasing glucose utilization in the liver. Normally, when circulating glucose concentrations are elevated, such as after a meal, glucose is taken up into hepatocytes and is phosphorylated to glucose-6-phosphate by the rate-limiting enzyme, glucokinase. Glucose-6-phosphate then enters the glycolytic pathway.

Therefore, it would be logical that leptin might reduce blood glucose levels by increasing its uptake and utilization in the liver. However, it has been observed that key regulatory enzymes of the glycolytic pathway, such as glucokinase, pyruvate kinase and pyruvate dehydrogenase complex, are down-regulated by leptin administered *in vivo* (166, 172, 250). An increase in glucose oxidation by the liver, therefore, does not explain the reduction in plasma glucose observed after leptin administration *in vivo*.

A reduction in glucose flux through gluconeogenic pathways could also lead to reduced hepatic glucose production and contribute to reducing plasma glucose concentrations. However, in the absence of leptin signalling and in the presence of profound insulin resistance and hyperglycemia, gluconeogenesis was reduced in hepatocytes isolated from *fa/fa* rats (295, 296). Furthermore, expression of two key enzymes of gluconeogenesis, phosphoenolpyruvate carboxylase (PEPCK) and glucose-6-phosphatase (Glc-6-Pase), were both reduced in livers of *ob/ob* mice (297), demonstrating that in the absence of leptin, gluconeogenesis is down-regulated. Conversely, mRNA expression of PEPCK and Glc-6-Pase is upregulated following leptin infusion *in vivo* (172, 250, 251) and leptin treatment of hepatic cells (119) and perfused liver (292). Therefore, leptin increases rather than decreases gluconeogenesis as was expected. Perfusion of fasted lean rat livers with leptin led to increased rates of hepatic glucose production and this was attributed to an increase in lactate-induced gluconeogenesis (279). Activation of PEPCK by leptin may be mediated through IRS-2 (292). These studies demonstrate that rather than suppressing gluconeogenesis, leptin up-regulates this process, which seemingly contradicts its role in reducing plasma glucose.

Another mechanism that may explain a reduction in plasma glucose concentrations following leptin administration in *ob/ob* mice is suppression of glycogenolysis. Despite an anti-insulin effect of leptin on gluconeogenesis, leptin exerts an insulin-mimicking effect on glycogenolysis, albeit there exists some conflicting reports in this area. A five-hour intravenous infusion of leptin into

wild-type mice reduced hepatic glycogen content (108). Transgenic skinny mice expressing leptin in the liver displayed marked decreases in hepatic glycogen stores (298). Despite these reports, other studies have indicated that leptin inhibits glycogenolysis and preserves glycogen stores (279, 286). In the livers of rats fasted for 20 h, leptin perfusion suppressed epinephrine-stimulated glycogenolysis (279). Co-perfusion with insulin did not further inhibit the actions of epinephrine suggesting that a single shared pathway may explain this insulin-like effect. However, studies in isolated rat hepatocytes demonstrated differential regulation of glycogen metabolism by leptin and insulin with regards to duration of stimulation and mechanism of activation (286). While insulin increased glycogen synthesis during a 3-h incubation, a preincubation period with leptin was required to elicit a similar effect, suggesting that perhaps leptin and insulin have overlapping but independent mechanisms for increasing glycogen synthesis. A mechanism by which leptin increases glycogen stores may be via the inhibition of glycogen phosphorylase, an enzyme that when activated, increases glycogen degradation (286). Other studies suggest that activation of glycogen synthesis via increased expression and activity of glycogen synthase kinase 3 may also play a role (283, 299). Thus, leptin-mediated increases in glycogen synthesis may be a restorative measure to ensure adequate glycogen stores during refeeding following a period of fast. From these studies, it would appear that leptin suppresses glycogenolysis and preferentially upregulates enzymes involved in glycogen synthesis.

In summary, leptin reduces glycolysis but increases gluconeogenesis and glycogen synthesis. The partitioning of glucose flux in favor of gluconeogenesis at the expense of glycolysis may dictate the preference of fuel utilization by leptin. The balance between these two pathways determines the availability of malonyl-CoA, an important molecule in the delegation of free fatty acids into β-oxidation or triglyceride formation. The leptin-induced intrahepatic redistribution of glucose through gluconeogenic rather than glycolytic processes may limit the availability of glucose designated for glycolysis and instead encourage the

oxidation of fatty acids. Since leptin is released from adipose stores and is a signal of nutritional and lipid abundance, preferential oxidation of fatty acids would limit their formation of triglycerides, thus serving to protect the liver from excessive accumulation of fat deposits and development of hepatic steatosis (157, 250). Therefore, although reductions of glycolysis, upregulation of gluconeogenesis, and preservation of glycogen stores may not fully explain a reduction in plasma glucose concentrations, these leptin-induced alterations may be important in the overall regulation of glucose metabolic fluxes in the liver. Irrespective of the intrahepatic distribution of glucose, the net effect of leptin appears to be a decrease in hepatic glucose production (172, 250), which may contribute to the overall reduction in plasma glucose concentrations.

1.11. Leptin Resistance

Although leptin administration has been demonstrated to reduce food intake and reverse obesity in leptin-deficient *ob/ob* mice (60), the relevance of this genetic model to the treatment of human obesity remains to be determined. A few rare cases of mutations in the leptin and leptin receptor genes have been described (143, 144, 146, 300, 301) although most human obesity is not accounted for by defects in these genes (302). Two cousins and another unrelated child, all of Pakistani origin have been identified with a homozygous frame-shift mutation in the leptin gene (ΔG133) resulting in profound obesity (144, 146). A missense mutation in the leptin gene in members of a family of Turkish origin results in a similar obese phenotype (143, 301). Although these single gene mutations are rare in humans, these subjects may be the most receptive to recombinant leptin therapy (144, 147). Heterozygosity for the ΔG133 frameshift mutation has also been identified in 13 subjects from three unrelated families of Pakistani origin (303). In these individuals, lower leptin levels are accompanied by an increased prevalence of obesity in comparison to their relatives that are homozygous for the wild-type *ob* gene. In this subgroup, it may be worthwhile to therapeutically treat with leptin in attempts to restore appropriate levels of leptin

function. Only one example of a mutation in the leptin receptor gene in humans has been reported in three siblings of Kabilian origin with morbid obesity (300). A homozygous mutation in the leptin receptor gene results in truncation of the leptin receptor and absence of both the transmembrane and intracellular domains of ObRb. These mutations, although rare, provide tremendous insight into the pathology incurred by the absence of functional leptin signalling.

Most obesity in humans is associated with increased serum leptin levels leading to the suggestion that obese humans may be leptin resistant (304, 305). Clinical trials in which leptin was administered to a cohort of obese individuals produced dismal results, with maximal doses of leptin eliciting only minor reductions in body weight (306, 307). In humans, increasing obesity induces a marked rise in leptin mRNA and protein levels in adipose tissue and a corresponding rise in circulating leptin levels that accurately reflects the amount of body fat gain (73, 75). However, despite increased leptin levels, animals fed a high fat diet became obese without decreasing their caloric intake, suggesting that a high content of dietary fat changes the body's response to the actions of leptin (74, 308). Similar to hyperinsulinemia and insulin resistance, hyperleptinemia may be indicative of leptin resistance. Leptin resistance at the level of the pancreatic islet may lead to deregulation of the adipoinsular axis, resulting in hyperinsulinemia. Inappropriate response to leptin in muscle, liver and fat may contribute to the hyperglycemia and hyperlipidemia often associated with obesity (309, 310). Potential mechanisms for leptin resistance include defects in transport of leptin across the blood-brain barrier preventing activation of leptin response neurons, or central and peripheral impaired leptin signalling in leptin sensitive organs.

Analysis of leptin concentrations in the cerebrospinal fluid of obese and non-obese humans revealed similar leptin levels, suggesting that leptin enters the brain by a saturable transport system and a defect in this transport limits leptin activity in the obese state (311, 312). Prevention of leptin entry in the brain would explain the development of central resistance to peripherally but not ICV-

administered leptin in a rodent model of diet-induced obesity (313). Leptin signalling through STAT3 in hypothalamus was preserved by ICV leptin treatment whereas peripheral leptin injection did not activate STAT3 (314). Elevated levels of triglycerides has been implicated as a potential cause of impaired leptin transport across the BBB (315).

Leptin resistance may ensue from defective physiological down-regulation of its own signal. Leptin interacts with SOCS in a negative feedback regulatory system. Leptin increases expression of SOCS mRNA in hypothalamus of *ob/ob* mice but not *db/db* mice (316). SOCS, in turn, down-regulates the leptin signal by inhibiting JAK phosphorylation. Inappropriately high levels of SOCS-3 activity may potentially induce leptin resistance. SOCS-3 mRNA is elevated in the hypothalamus of the Agouti mouse, a model of obesity that is characterized by hyperleptinemia and resistance to both central and peripheral leptin administration (316). SOCS inhibition of leptin mediated lipopenic effects is demonstrated in islets and in adipocytes (317). Therefore, elevations in SOCS may lead to leptin resistance.

A similar negative feedback mechanism exists between leptin and a protein tyrosine phosphatase (PTP), Src homology 2 containing phosphatase, 2 (SHP2). Upon leptin stimulation, SHP2 becomes tyrosine phosphorylated and activated. SHP2, in turn, reduces phosphorylation of JAK2 and STAT3 (318, 319). These findings indicate SHP2 as a negative regulator of STAT3-mediated gene induction and implicates a defect in this signalling pathway as a potential mechanism of leptin resistance.

In recent years, several lines of evidence have converged on another PTP, protein tyrosine phosphatase 1B (PTP1B) as one of the most important negative regulators of leptin signalling. Originally discovered as a key inhibitor of insulin signalling (Fig. 1.2), PTP1B is now postulated to additionally regulate leptin sensitivity and body weight. Mice in which the PTP1B gene is disrupted ($PTP1B^{-/-}$) exhibited lower circulating concentrations of glucose and insulin and enhanced glucose clearance, illustrating improved insulin sensitivity (320, 321).

These mice were hypersensitive to insulin, and displayed increased IR phosphorylation in liver and muscle. In addition, PTP1B$^{-/-}$ mice resisted developing obesity when fed a high fat diet, attributed largely to increased energy expenditure (320, 321), thus hinting at the possibility that leptin sensitivity is increased. Subsequently it was demonstrated that *ob/ob* mice lacking PTP1B gained less weight, and had reduced adiposity and increased basal metabolic rates in comparison to *ob/ob* mice with PTP1B (322). PTP1B interfered with leptin signalling by inhibiting JAK2 phosphorylation (322, 323). Furthermore, gold-thioglucose-induced destruction of ObR-expressing cells in the hypothalamus of PTP1B$^{-/-}$ resulted in reduced weight gain compared to wild-type mice (323). However, the weight gain was only half as much as in wild-type control mice despite equal consumption of food suggesting that leptin may interact with peripheral leptin receptors in muscle fat and/or liver. Based on these studies, PTP1B is an ideal candidate for a leptin resistance factor and inhibition of its activity may prove beneficial in the treatment of both obesity and diabetes.

Other than dietary factors, genetic influences may also predetermine an individual's susceptibility to developing leptin resistance. It has recently been reported that DNA sequence variations in the genes for leptin and the leptin receptor are associated with the degree by which physical activity improves insulin sensitivity in a non-diabetic individual. The screening of polymorphisms in the leptin and leptin receptor genes in individuals who are predisposed to developing insulin resistance may be beneficial in determining the benefit of physical activity as treatment for the prevention of diabetes (324).

1.12. Leptin – the Next Step

The association of obesity and diabetes has been recognized for decades. Yet, the common thread that links these two diseases remains to be identified. Increasing adiposity is attributed to both insulin resistance and insulin secretory defects. While the debate continues on which is the principle cause of diabetes, the question arises as to whether there exists a common defect that could give rise

to both insulin resistance and impaired insulin secretion. One possibility is leptin. Rodent models of congenital leptin deficiency exhibit both obesity and diabetes (63). In humans with an analogous defect, the absence of leptin is associated with hyperinsulinemia (144, 146, 147) and hyperglycemia (143). While leptin is clearly capable of reducing body weight, it can also reduce plasma glucose and insulin levels in *ob/ob* mice prior to changes in body mass (59). Collectively, these observations hint that defective leptin action may be the common abnormality that precipitates obesity-associated diabetes. One mechanism by which leptin action may be impaired is the enhanced expression of PTP1B, a phosphatase that potentially inhibits both insulin and leptin signalling. Deregulation of PTP1B function may lead to reduced sensitivity to both hormones and predispose the development of obesity and diabetes. An understanding of the mechanisms by which humans become resistant to leptin with increasing adiposity may provide alternative targets for therapeutically restoring the actions of leptin. The following studies will investigate mechanisms by which leptin can affect glucose homeostasis independently of body weight changes, focusing specifically on the inhibition of insulin secretion from pancreatic β cells and improvement in insulin sensitivity in non-central tissues. In addition, these studies will evaluate the role of PTP1B as a potential mediator of leptin and insulin resistance. It is hoped that these studies will enhance our understanding of the role of leptin in glucose homeostasis and the pathophysiology of obesity-associated diabetes.

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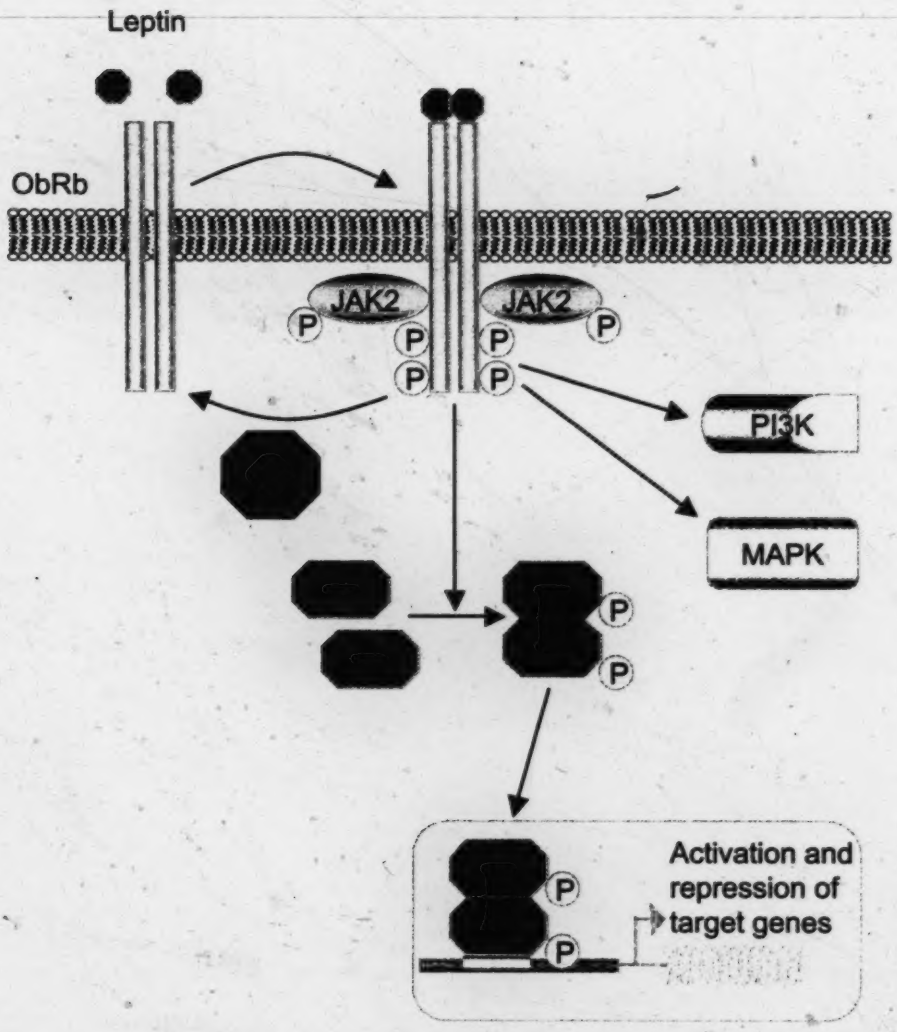

Figure 1.1. Leptin signalling pathway. Leptin binds to the long form of the leptin receptor (ObRb), leading to phosphorylation of Janus kinase 2 (JAK2). Phosphorylated JAK2 then phosphorylates ObRb and signal transducers and activators of transcription (STAT). Phosphorylation of STAT results in its dimerization and translocation to the nucleus where STAT induces both activation and repression of target genes. Leptin also activates phosphatidylinositol-3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) signalling via pathways that are less understood. Protein tyrosine phosphatase 1B (PTP1B) downregulates leptin signalling by dephosphorylating JAK2.

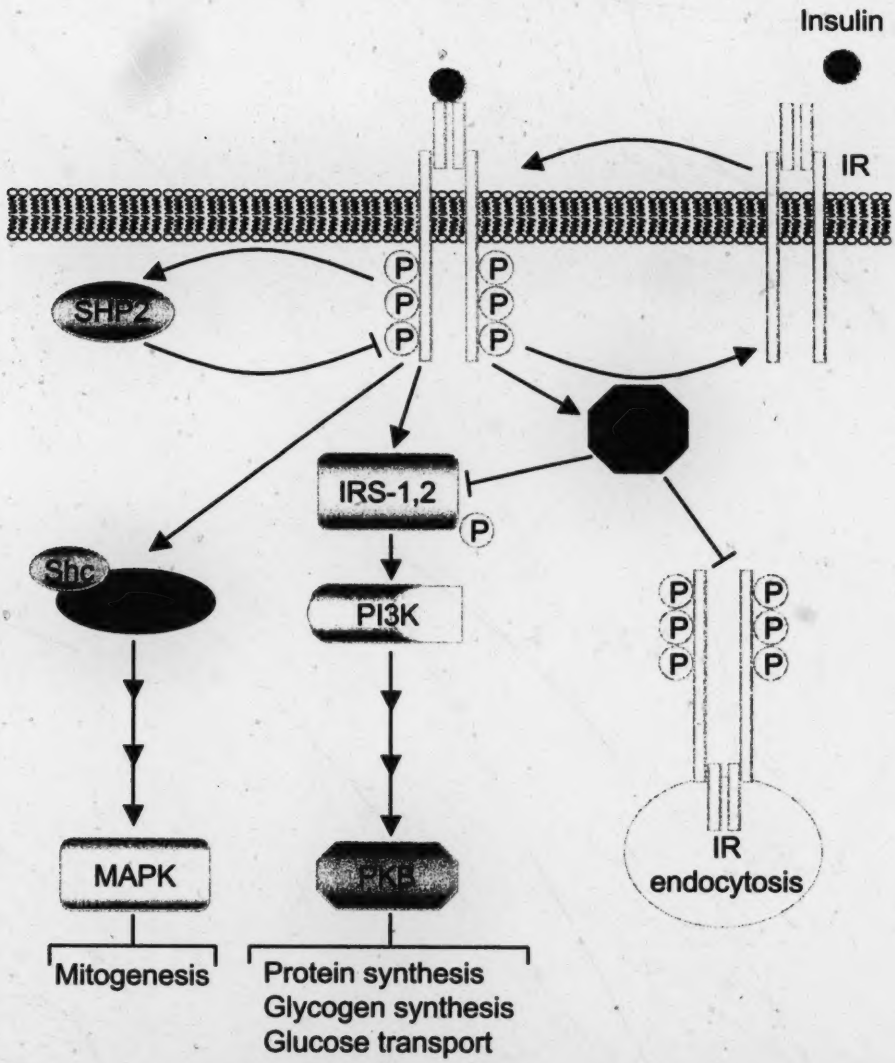

Figure 1.2. Insulin signalling pathway. Upon binding of its ligand, the insulin receptor (IR) autophosphorylates and induces activation of its kinase activity. Recruitment and phosphorylation of the insulin receptor substrates-1 and -2 (IRS-1,2) induces activation of phosphatidylinositol-3-kinase (PI3K) through binding of the p85 subunit and activation of the catalytic p110 subunit. PI3K activation induces downstream effectors including protein kinase B (PKB) leading to modification of glucose transport and glycogen and protein synthesis. Insulin can also engage $p21^{ras}$ through tyrosine phosphorylation of Shc to signal via mitogen-activated protein kinase (MAPK), and thereby activate mitogenic pathways. Activated IR increases protein tyrosine phosphatase 1B (PTP1B) activity, which inhibits insulin signalling by dephosphorylating membrane-bound or endocytosed IR and IRS-1. Similarly, IR activates another phosphatase, Src homology 2 containing phosphatase 2 (SHP2), which also dephosphorylates IR and downregulates insulin signalling.

CHAPTER 2

LEPTIN REDUCES GLUCOSE TRANSPORT AND CELLULAR ATP LEVELS IN INS-1 β CELLS

2.1. Introduction

Leptin, the product of the *ob* gene is produced primarily by adipose tissue, and relays information about adipocyte metabolism and body weight to the appetite centers in hypothalamic regions of the brain (1). Mice that harbor genetic mutations rendering them incapable of expressing functional leptin protein (*ob/ob*) or leptin receptors (*db/db*) are characterized by the development of obesity and type 2 diabetes. These mice are hyperphagic, insulin resistant and have high circulating blood insulin and glucose levels. This phenotype in *ob/ob* mice is rapidly normalized by leptin treatment (2-5). While the effects of leptin on satiety and energy expenditure appear to be centrally mediated, leptin action has also been reported in peripheral tissues, such as liver, skeletal muscle, adipose tissue, and pancreas (6, 7). The long isoform of the leptin receptor (ObRb) is expressed in pancreatic β cells (8) and although the direct effects of leptin on insulin secretion are still controversial (9-11), leptin has been reported to inhibit insulin secretion from isolated islets from *ob/ob* and wild-type mice and tumour-derived β cell lines (12-17).

Leptin has been shown to activate ATP-sensitive K^+ (K_{ATP}) channels, which play a central role in the control of insulin secretion from pancreatic β cells (14, 18, 19). Elevation in ATP relative to ADP by glucose and subsequent closure of K_{ATP} channels results in the reduction of K^+ efflux from the cells, leading to depolarization of the plasma membrane and activation of

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voltage-dependent Ca^{2+} channels (VDCC). The consequent rise in intracellular Ca^{2+} levels results in insulin secretion (20). Electrophysiological studies on *ob/ob* β cells and on the rat insulinoma cell line, CRI-G1, showed that the application of leptin hyperpolarizes the cells as a result of opening the K_{ATP} channels (14, 18, 19), thereby inhibiting insulin secretion. Leptin-induced K_{ATP} channel activation in the CRI-G1 β cell line appears to involve signalling through phosphoinositide 3-kinase (PI3K) (19, 21) and may involve disruption of the actin cytoskeleton (22). Since K_{ATP} channels are sensitive to changes in ATP levels (23), we sought to determine whether leptin might also open K_{ATP} channels by reducing the intracellular concentration of ATP ($[ATP]_i$). Furthermore, since K_{ATP} channels are sensitive to the ATP/ADP ratio, which in turn is governed by the availability of glucose for metabolism, we investigated whether leptin could affect glucose availability by reducing glucose transport in INS-1 cells, an insulin-secreting, tumour-derived, pancreatic β cell line, previously shown to be responsive to leptin (16).

2.2. Materials and Methods

Cell culture

INS-1 cells were kindly provided by Professor Claes Wollheim, Geneva, Switzerland and Dr. Marc Prentki, Montreal, Canada. Cells were cultured at 37°C in humidified air containing 5% CO_2/95% O_2 in RPMI 1640 medium, pH 7.4, supplemented with 2 mM L-glutamine, 10% w/v fetal bovine serum (FBS), 100 U/ml penicillin, 100 μg/ml streptomycin, 11 mM glucose, and 50 μM β-mercaptoethanol (all reagents from Life Technologies Inc., Grand Island, NY).

Rat hepatocyte isolation and culture

Hepatocytes were isolated by collagenase perfusion of the livers of male Sprague Dawley rats (200-300 g; Biological Sciences Animal Services, University of Alberta, Edmonton, AB, Canada) fed *ad libitum*, as previously described, with modifications (24). Animal studies were conducted in accordance with and under the approval of the Health Sciences Animal Policy and Welfare Committee, University of Alberta, Edmonton, AB, Canada. In brief, the hepatic portal vein was cannulated and perfused with a Ca^{2+}-free solution, pH 7.4 (142 mM NaCl, 6.7 mM KCl, 10.1 mM Hepes, 5.5 mM NaOH), prior to perfusion with Type IV collagenase , pH 7.6, (Sigma-Aldrich Canada Inc., Oakville, ON, Canada). Cells were isolated, trypan blue (Sigma-Aldrich) stained for viability, then cultured for 24 h in M199 media, pH 7.4, (Sigma-Aldrich) supplemented with 0.2% wt/v BSA (Sigma-Aldrich) prior to glucose transport studies.

Measurement of intracellular ATP

INS-1 cells were cultured in 96 well black Optilux-Iso microplates (Becton Dickinson and Co., Sparks, MD) for 24 – 48 h in RPMI medium, pH 7.4, supplemented as described above. Cells were washed twice in bicarbonate buffer, pH 7.4, (120 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $MgCl_2$, 1mM Na_2HPO_4, 24 mM $NaHCO_3$, 1 mg/ml BSA) and maintained in this buffer for 4 h prior to ATP assay. After 3 h and 15 min of incubation, cells were treated with 10

ng/ml recombinant human leptin (PeproTech Inc., Rocky Hill, NJ) for the remaining 45 min. Prior to the ATP assay, cells were challenged with 5 mM glucose in bicarbonate buffer for 1-30 min. Cells were lysed with a somatic cell ATP releasing reagent (Sigma-Aldrich) and immediately placed on ice. Intracellular ATP was determined by a luciferin/luciferase method using an ATP bioluminescent assay kit (Sigma-Aldrich). Luminescence was determined using Wallac Trilux 1450 Microbeta liquid scintillation and luminescence counter (Wallac Oy, Turku, Finland). Use of the 96-well microplate limited crosstalk between samples to 0.002%. The instrument was preset to integrate the amount of light produced over a 5 second interval without and initial delay. Standard curves generated from ATP standards (Sigma-Aldrich) displayed linearity in the range of $1x10^{-12}$ to $1x10^{-8}$ mol ATP.

Patch-Clamp Experiment

The perforated patch technique was used to measure whole cell currents from INS-1 cells while maintaining the intracellular signalling environment intact. Amphotericin-B (Sigma) was dissolved in dimethylsulfoxide (DMSO; 40mg/ml) and diluted in the pipette solution immediately before use to yield a final concentration of 80μg/ml. Pipettes were back-filled with this amphotericin-containing solution. The pipette solution consisted of the following (in mM): KCl 10, Kaspartate 130, HEPES 10, $MgCl_2$ 1.4, EGTA 1, glucose 10. The pH of the solution was adjusted to 7.4 with KOH. Patch pipettes were pulled using borosilicate glass (G85150T, Warner Instrument Corp., Hamden, CT) to yield pipettes with a tip resistance of 2-6MΩ when filled with pipette solution. Once a GΩ seal was obtained, series resistance was monitored. A stable perforation access resistance of less than 20MΩ was deemed acceptable. All whole-cell experiments were performed on single cells and recorded in voltage-clamp mode using an Axopatch 200B amplifier and Clampex 8.0 software (Axon Instruments, Foster City, CA). K_{ATP} channel current was elicited using a series of 480 ms voltage ramps from –90mV to –30mV, holding at –70mV. Currents were

sampled every 5 seconds at 2 kHz. Cells were initially perfused with control solution containing the following in mM: NaCl 140, HEPES 10, $CaCl_2$ 1, $MgCl_2$ 1.4, KCl 5, glucose 10. Intracellular ATP levels were depleted using a chemically-induced metabolic inhibition solution (MI) prepared by replacing glucose with 4mM NaCN and 5mM 2-deoxy-glucose (2-DOG) in the control solution. Regeneration of intracellular ATP was achieved by superfusing the cells with control solution. Control cell experiments were performed in the absence of leptin in all solutions. Experimental cells were exposed to 10ng/ml leptin for approximately 2-3 minutes prior to MI. Leptin was present in both control and MI solutions. Time required for K_{ATP} channel currents to recover 50% of the ATP-mediated inhibition relative to peak current obtained during MI versus that obtained in the control period was analyzed.

Measurement of glucose transport

Cells were cultured in 60 mm dishes (Becton Dickinson) to a confluency of ~80%. Cells were washed twice in a buffered solution, pH 7.4, (5.4 mM KCl, 1.8 mM $CaCl_2$, 1.2 mM $MgSO_4$, 10 mM Hepes, 137 mM NaCl, 0.2% wt/v BSA) and fasted in the absence of glucose and serum for 2 h. Cells were treated with 1-100 ng/ml recombinant human leptin (PeproTech Inc.) for various periods between 5 and 120 min. After a 2 h incubation period, cells were washed twice with the buffered solution without BSA. Cells were then exposed for 15 s to 4 min at 25°C, in the absence of leptin, to a 5 mM 3-O-methylglucose solution (pH 7.4)(Sigma-Aldrich), in which ^{3}H-3-O-methylglucose (Amersham Pharmacia Biotech, Inc. Baie d'Urfe, PQ, Canada) was added to a final specific radioactivity of 0.25 μCi/ml. Time 0 uptake was assessed by pretreating cells with 20 μM cytochalasin B (Sigma-Aldrich) for 15 min prior to glucose uptake. The radioactivity remaining in these lysates was considered background and subtracted from all time points. Transport was stopped by immersing the cells in an ice-cold 100 μM phloretin solution (Sigma-Aldrich). Cells were washed in phloretin solution to remove extracellular [^{3}H] and then lysed with a 0.5% v/v

triton X-100 solution (Sigma-Aldrich) and radioactivity was counted by a liquid scintillation and luminescence counter (Wallac Trilux 1450 Microbeta, Turku, Finland).

^{32}P loading and immunoprecipitation

Cells were serum starved for 2 h in RPMI media, pH 7.4, prior to being transferred into phosphate-free RPMI containing 10 μg/ml protease and phosphatase inhibitor cocktail (Sigma-Aldrich), 1 mM PMSF (Sigma-Aldrich) and 0.1% wt/v BSA, for metabolic labeling with ^{32}P (as orthophosphate; Amersham Pharmacia Biotech, Inc.). Cells were labeled with a specific radioactivity of 0.1 mCi ^{32}P/ml/per 10^8 cells for a period of 2 h at which point cells were treated with 10 or 100 ng/ml recombinant human leptin for 30 min and/or with 10 nM GLP-1 7-36NH_2 (Peninsula Laboratories Inc., Belmont, CA) for 10 min. Cells were lysed in lysis buffer (50 mM Hepes, 1% v/v Triton-X, 2 mM EDTA, 200 mM NaF, 10 mM $Na_2P_4O_7$, 1 mM PMSF, 1 mM Na_3VO_4, 10 μg/ml protease inhibitor cocktail), harvested and immunoprecipitated with 5 μg affinity purified rabbit GLUT2 antibody (Alpha Diagnostic Intl., San Antonio, TX) and protein A conjugated protein A/G (Pierce Chemical Company, Rockford, IL) overnight at 4°C. Immunoprecipitates were washed 3 times with lysis buffer and then boiled in sample buffer (1 M TrisCl, pH 6.8, 30% v/v glycerol, 5% v/v SDS, 0.1% wt/v bromophenol blue, 5% v/v β-mercaptoethanol). The resulting supernatants were run on a 10% SDS-PAGE gel. The gel was dried for the determination of the level of radioactivity of GLUT2 by phosphoimaging (STORM 840, Amersham Pharmacia Biotech, Uppsala, Sweden). Densitometric values were determined with Molecular Dynamics ImageQuant software (Amersham Pharmacia Biotech).

Membrane protein extraction

Cells grown on 100 mm plates (Becton Dickinson) were placed on ice and collected by scraping and centrifugation. Cells were resuspended in lysis buffer

(10 mM Tris, 1 mM PMSF), and homogenized with a dounce homogenizer. The homogenate was centrifuged at 4000 x g for 5 min at 4°C. The pellet was discarded and the supernatant was centrifuged at 110000 x g for 20 min at 4°C in a Beckman ultracentrifuge (SW41 rotor). Subsequently the supernatant was discarded and the pellet was resuspended in lysis buffer and again homogenized with a dounce homogenizer and ultracentrifuged at 110000 x g for 20 min at 4°C. The supernatant was aspirated and the remaining pellet was resuspended in phosphate-buffered saline containing 0.5% v/v 3-[(3-cholamidopropyl)dimethyammonio]-1-propanesulfonate hydrate (CHAPS) and 1 mM PMSF for 30 min at 4°C. The lysate was centrifuged at 15800 x g for 5 min at 4°C and the supernatant fraction was collected for determination of protein concentration by DC protein assay (Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada)

Western blot

Protein samples were run on 10% SDS-PAGE gel and transferred overnight at 4°C to nitrocellulose membrane (Osmonics Inc., Westborough, MA). The membrane was blocked in TBS-Tween, pH 7.4, (20 mM Tris, 150 mM NaCl, 0.1% v/v tween 20) containing 5% non-fat dry milk. Immunoblot was performed using affinity-purified rabbit GLUT2 antibody (Alpha Diagnostic Intl.) and rabbit IgG conjugated to HRP (Amersham Pharmacia Biotech, Inc). Protein was visualized by enhanced chemiluminescence (ECL western blotting system; Amersham Pharmacia Biotech, Inc.). The chemiluminescent signal was captured on Kodak BioMax MR film (Eastman Kodak Co., Rochester, NY).

Statistical analysis

The significance of group differences was evaluated by ANOVA analysis and Tukey post-hoc tests with Statview software (SAS Institutes Inc., Cary, NC) and values are presented as means ± standard error. $P<0.05$ was deemed significant.

2.3. Results

Effects of leptin on $[ATP]_i$ in INS-1 cells

INS-1 cells were pretreated with leptin (10 ng/ml) for 45 min, then challenged with 5 mM glucose for 1-30 min. In the basal state (no glucose challenge), there was no difference in ATP levels between vehicle- and leptin-treated cells. In the presence of 5 mM glucose, $[ATP]_i$ in INS-1 cells rose ~1.5-fold within 1 min of glucose stimulation ($P<0.0001$, $n=4$, 0 vs 1 min) and appeared to reach a plateau of ~2.2-fold after 10 min ($P<0.0001$, $n=4$, 0 vs 10 min)(Fig. 2.1). In contrast to controls, $[ATP]_i$ did not increase within 1 min ($P=0.8$, $n=4$, 0 vs 1 min) and only rose to ~1.5 fold after 5 min in the presence of leptin ($P=0.003$, $n=4$). ATP levels did not appear to reach a plateau of ~2.2-fold until 15-20 min of glucose stimulation in leptin-treated cells. Therefore, $[ATP]_i$ is significantly reduced in the presence of leptin in the first 10 min of glucose stimulation ($P<0.03$, $n=4$, control vs leptin treated cells at 1, 5, and 10 min).

Effects of leptin on whole-cell K_{ATP} channel currents in INS-1 cells

We examined the effects of ATP depletion/regeneration on the activation and subsequent recovery of whole-cell K_{ATP} channel currents in intact INS-1 cells. Intracellular ATP was depleted using a solution containing no glucose and the metabolic inhibitors sodium cyanide and 2-deoxyglucose (MI solution). Perfusion of INS-1 cells with the MI solution resulted in the rapid development of large whole-cell currents. These currents were inhibited by application of the K_{ATP} channel-specific sulphonylurea drug tolbutamide (100μM; Fig. 2.2A) as well as by reperfusion with control solution containing 10mM glucose (Fig. 2.2B). Increases in tolbutamide-sensitive current were recorded within 1-2 minutes of metabolic inhibition indicating a reduction in intracellular ATP levels and likely, a corresponding increase in K_{ATP} channel current. In cells treated with leptin (10ng/ml), the time required for ATP levels to regenerate sufficiently to inhibit 50% of the MI-induced K_{ATP} channel current was significantly increased,

from 212 ± 22 seconds (n =7 cells) to 484 ± 55 seconds (n =8 cells, $P<0.01$, Fig. 2.2C).

Effects of leptin on glucose transport in INS-1 cell

Glucose transport was assessed by measuring the uptake of ^{3}H-3-O-MG, a transported, but non-metabolizable hexose substrate. Transport of 3-O-MG was a saturable process with increasing exposure time to the substrate, reaching a maximum by 4 min (3.2 ± 0.2 nmol/μg protein)(Fig. 2.3A). The rate of 3-O-MG uptake remained linear within the first 45 s. Leptin (≥ 1 ng/ml) reduced the rate of glucose transport in INS-1 cells in a dose-dependent fashion. After 15 s, 3-O-MG uptake in cells pretreated with 10 ng/ml leptin for 30 min only reached 64 ± 3% ($P<0.0001$, $n=5$) of the level reached by vehicle-treated cells (Fig. 2.3B). The effect of leptin on glucose transport was acute with a significant reduction by 20 min of leptin treatment (33 ± 11%, $P=0.03$, $n=4$) (Fig. 2.3C).

Transporter regulation by leptin and GLP-1

GLUT2 is the primary glucose transporter responsible for shuttling glucose into β cells (25). To determine whether the acute effect of leptin on glucose transport may be due to changes in phosphorylation of the transporter, we assessed changes in phosphorylation of GLUT2. Western blot analysis showed no difference in GLUT2 membrane expression levels following either GLP-1 or leptin treatment for 30 min (Fig. 2.4A). To look at changes in the phosphorylation state of GLUT2, INS-1 cells were labeled metabolically with ^{32}P, then treated with GLP-1, an insulin secretagogue, previously demonstrated to alter GLUT2 phosphorylation states (26), and/or leptin, followed by GLUT2 immunoprecipitation. Treatment of cells with GLP-1 (10 nM) for 10 min increased GLUT2 phosphorylation by ~1.5-fold. While leptin alone had no effect on GLUT2 phosphorylation, preincubation with leptin (10 or 100 ng/ml) for 30 min completely abolished GLP-1 stimulated phosphorylation of GLUT2 (Fig. 2.4B).

Effects of leptin on glucose transport in isolated hepatocytes

Since hepatocytes express GLUT2 similarly to INS-1 cells, we investigated whether the effects of leptin on glucose transport in INS-1 cells could be mimicked in rat hepatocytes. In rat hepatocytes treated with leptin (10 ng/ml) for 15 min glucose transport was 66±2% (P=0.01, n=4) of vehicle-treated cells (Fig. 2.5). This finding suggests that leptin might specifically target the GLUT2 transporter in both β cells and hepatocytes.

2.4. Discussion

Leptin has been reported to activate K_{ATP} channels in β cells by signalling through the PI3K pathway (19, 21) and by mediating the downstream disruption of the actin cytoskeleton (22). From this present study, we propose that leptin may additionally target the glucose metabolic pathway, reducing the elevation in ATP levels that normally results from glucose metabolism, and thereby activating K_{ATP} channels. In the absence of gluocse the $[ATP]_i$ was not different between vehicle and leptin-treated cells. However, leptin attenuated glucose-induced increases in $[ATP]_i$ by ~25% within the first 10 min of glucose stimulation. Gradually lowering the ATP/ADP ratio in mouse islets by increasing concentrations of respiratory chain and glycolytic inhibitors has been shown to progressively inhibit secretion by opening K_{ATP} channels (Detimary *et al.* 1994). We have tested the ability of INS-1 cells to regenerate ATP and therefore increase the ATP/ADP ratio after a brief period of metabolic inhibition . Tolbutamide-sensitive currents were rapidly elicited during metabolic inhibition and reduced by inhibitor washout and subsequent reperfusion with glucose. The effect of a physiological concentration of leptin was to significantly prolong K_{ATP} channel activity following metabolic inhibition, an observation that may be attributed to a delayed increase in the ATP/ADP ratio observed in this study. Therefore, our finding that leptin attenuates the production of intracellular ATP from glucose metabolism and as a result prolongs the activity of K_{ATP} channels may explain, in part, the reduction of glucose-stimulated insulin secretion observed in the presence of leptin (Harvey *et al.* 1997, Kieffer *et al.* 1997, Cases *et al.* 2001).

The observation that leptin reduces glucose-stimulated elevations in $[ATP]_i$ suggests that leptin inhibits a substrate-driven pathway that normally results in the production of ATP. Leptin potentially reduces ATP concentrations in β cells by increasing the activity of uncoupling proteins (UCPs). Zhou *et al.* demonstrated that overexpression of leptin in mice by adenoviral delivery resulted in a 6-fold increase in the levels of UCP-2 mRNA in islets (27). An elevation of UCP-2 mRNA was also observed *in vitro* in islets cultured with recombinant

leptin (27). Alternatively, given that changes in glucose availability give rise to changes in $[ATP]_i$, leptin potentially decreases $[ATP]_i$ by reducing glucose transport into β cells. Indeed, in our study, we found that leptin dose-dependently reduced glucose transport in INS-1 cells. Although a reduction of glucose transport is proposed to translate to a reduction in $[ATP]_i$, it is generally accepted that the rate of glucose transport greatly exceeds the rate of glucose phosphorylation by glucokinase, and therefore, glucose metabolism, and not transport is the rate-limiting step (28). However, it has been argued that when the expression or function of glucose transporters is significantly reduced, the access of glucose to glucokinase may be limiting, and therefore the rate of glucose transport can be indicative of the rates of glucose metabolism and insulin secretion (29-31). In our studies, we measured glucose transport at 0.5 mM (data not shown) and 5.0 mM 3-O-MG, substrate concentrations below the level (>5.5 mM) that would normally render glucokinase as the rate-limiting step of glucose metabolism (32, 33). Therefore, leptin-mediated reductions in glucose transport could translate to less substrate available for metabolism to ATP.

The acute effect (20 min) of leptin on glucose transport suggests that these changes are unlikely to be mediated by changes in GLUT2 protein levels. Furthermore, unlike GLUT4, GLUT2 mediated glucose transport is not thought to be regulated by translocation between intracellular vesicles and the plasma membrane (34). In agreement, our western blot analysis of membrane proteins from leptin-treated INS-1 cells showed approximately equal abundance of GLUT2. Therefore, changes in protein levels do not appear to explain the leptin-mediated reductions in glucose transport. It has been postulated that GLUT2 activity can be affected by phosphorylation of the transporter (26). Both forskolin and GLP-1 have previously been shown to induce protein kinase A-dependent phosphorylation of GLUT2 on specific serine and threonine residues of the carboxyl-terminal tail of the transporter, thus altering transport activity (26). Although changes in the phosphorylation state of GLUT2 was one mechanism by which glucose transport activity could be altered, leptin on its own, did not alter

GLUT2 phosphorylation in our study. However, leptin reduced the GLP-1 stimulated increase in GLUT2 phosphorylation, suggesting that leptin may play an antagonist role to GLP-1. The decrease in phosphorylation may have resulted from leptin reducing ATP levels which may lead to a reduced production of cAMP upon GLP-1 treatment. These findings support observations that leptin inhibited insulin secretion from INS-1 cells under conditions in which intracellular cyclic AMP (cAMP), was increased (16). These results are similar to the interaction between leptin and GLP-1 on preproinsulin mRNA expression (35). Under conditions that did not support leptin-mediated reductions of insulin mRNA levels, the presence of 10 nM GLP-1 stimulated the expression of preproinsulin mRNA 2-fold, and this stimulation was abolished by 6.25 nM leptin (35). Therefore, although a reduction in GLP-1 induced GLUT2 phosphorylation by leptin may play a role in the ability of leptin to reduce insulin secretion stimulated by an increase in cAMP, this mechanism does not appear to explain the effect of leptin alone on inhibiting insulin secretion *in vivo* (14).

Through its actions on the β cell, leptin plays a key role in the regulation of glucose homeostasis by reducing insulin release. The liver has been implicated as an additional target for leptin regulation of glucose metabolism. *In vivo*, leptin reduces high circulating blood glucose levels (2-5) and central or i.v. infusion of leptin to rats enhances hepatic insulin sensitivity and increases whole-body glucose utilization (36-39). The presence of leptin receptors on hepatocytes support the observations of leptin action on the gene expression of gluconeogenic and glycogenic enzymes and leptin interaction with the insulin signalling cascade in hepatic cell lines (40-46). Since liver and β cells express GLUT2, we determined whether leptin might also diminish glucose transport in hepatocytes. Similar to effects observed in β cells, 3-O-MG levels in leptin-treated cultured rat hepatocytes were significantly reduced compared to controls. Since 3-O-MG is non-metabolizable, any changes in cellular concentrations reflect changes in transport. Given that a reduction in glucose transport into hepatocytes is a reflection of a reduced intrinsic activity of the transporter, glucose transport out of

hepatocytes is proposed to be similarly lowered by leptin. Therefore, *in vivo*, a combination of effects on glucose transport and on gluconeogenic and glycogenolytic pathways by leptin may culminate in an overall result of reduced hepatic glucose output, thereby lowering blood glucose levels.

In summary, we have identified a proximal pathway by which leptin may reduce insulin secretion in pancreatic β cells. The inhibition of insulin secretion by leptin might, in part, explain the reduction in plasma insulin when leptin is administered to *ob/ob* mice with hyperinsulinemia. Interestingly, leptin-mediated reductions in glucose transport may also occur in hepatocytes. This common mechanism of action in β cells and hepatocytes could contribute to the reduction of both plasma insulin and glucose observed *in vivo* following leptin treatment. Further studies are required to determine the exact mechanism by which leptin reduces glucose transport.

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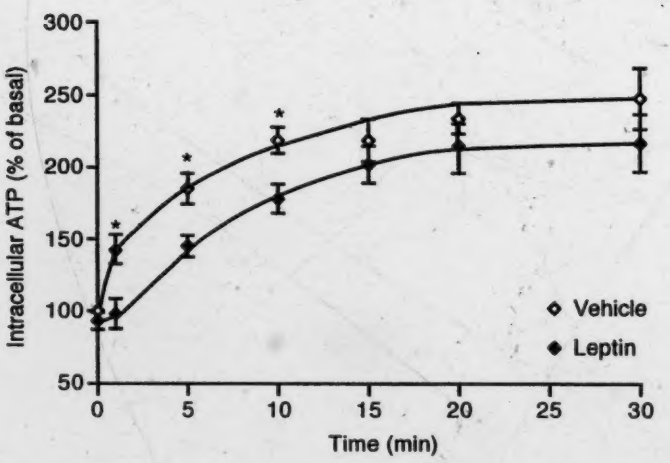

Figure 2.1. Effect of leptin on intracellular ATP levels in INS-1 cells. After a 4 h period of fasting in glucose free media and pretreatment with 10 ng/ml leptin for 45 min, INS-1cells were exposed to 5 mM glucose for 1 to 30 min. Intracellular ATP values are expressed as mean percent ± SEM of ATP levels in non-glucose stimulated, vehicle-treated cells (comparison between vehicle and leptin-treated cells at each time point, *$P<0.05$, n=4)

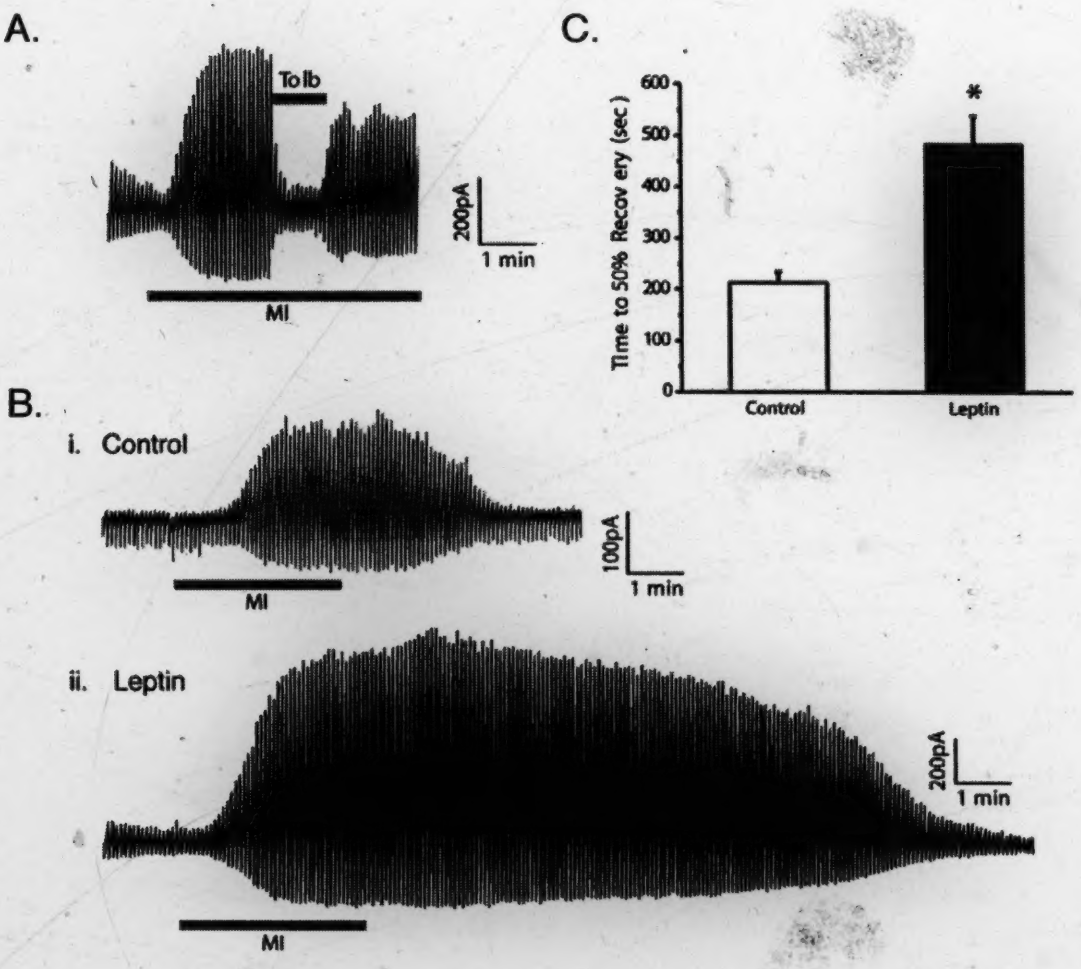

Figure 2.2. Effects of leptin on ATP-mediated closure of K_{ATP} channels following ATP depletion. A. Whole-cell K_{ATP} channel current measured using the perforated patch-clamp technique in response to ATP depletion during metabolic inhibition (MI). MI-induced currents were rapidly and reversibly inhibited by the K_{ATP} channel-selective inhibitor tolbutamide (100 μm). B. Representative increases in whole-cell K_{ATP} channel current in response to MI and subsequent recovery in the absence (i) and presence (ii) of 10 ng/ml leptin. C. Grouped data from whole-cell experiments represented in B. *denotes statistical significance ($P<0.01$. Student's t-test) vs control (no leptin)

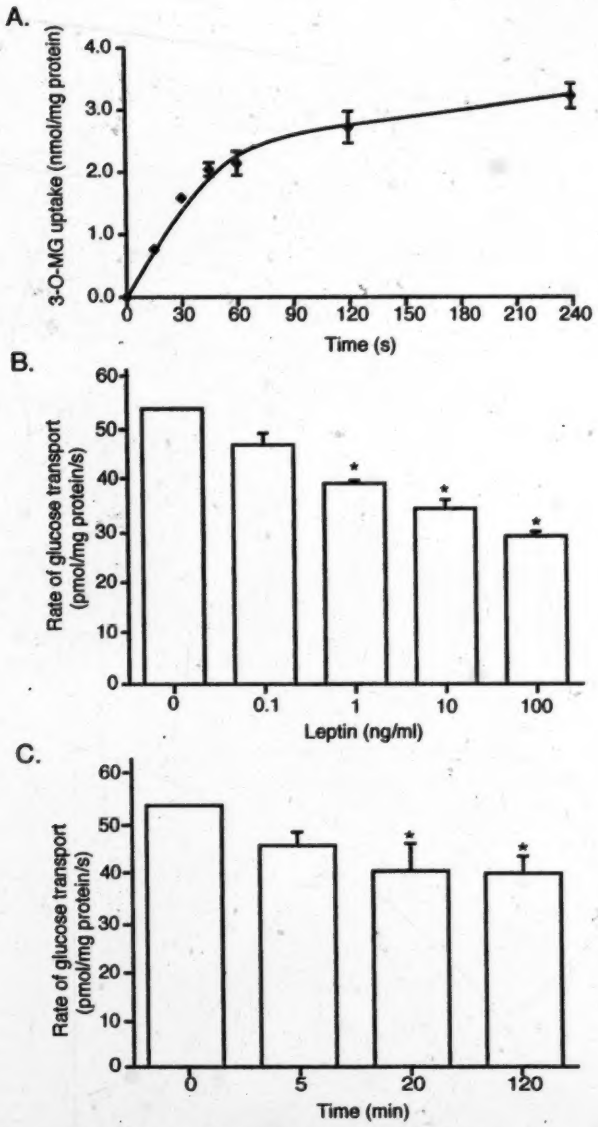

Figure 2.3. Effects of leptin on glucose transport in INS-1 cells. A. 3-O-MG transport as a function of time in INS-1 cells. Cells were exposed to 5 mM ^{3}H-3-O-MG for up to 4 min. Time 0 uptake was assessed by pretreating cells with 20 μM cytochalasin B for 15 min prior to glucose uptake. The radioactivity remaining in these lysates was considered background and was subtracted from all time points. Results are expressed as mean ± SEM. B. Dose response to leptin on glucose transport in INS-1 cells. Prior to exposure to ^{3}H-3-O-MG for 15 s, INS-1 cells were treated with leptin for 30 min (values compared to 0 ng/ml; *P<0.0001, n=5). C. Time response of leptin on glucose transport in INS-1 cells. Prior to exposure to ^{3}H-3-O-MG, INS-1 cells were treated with 10 ng/ml leptin for the durations indicated (values compared to 0 min; *P<0.005, n=4). Values in B and C are presented as mean + SEM.

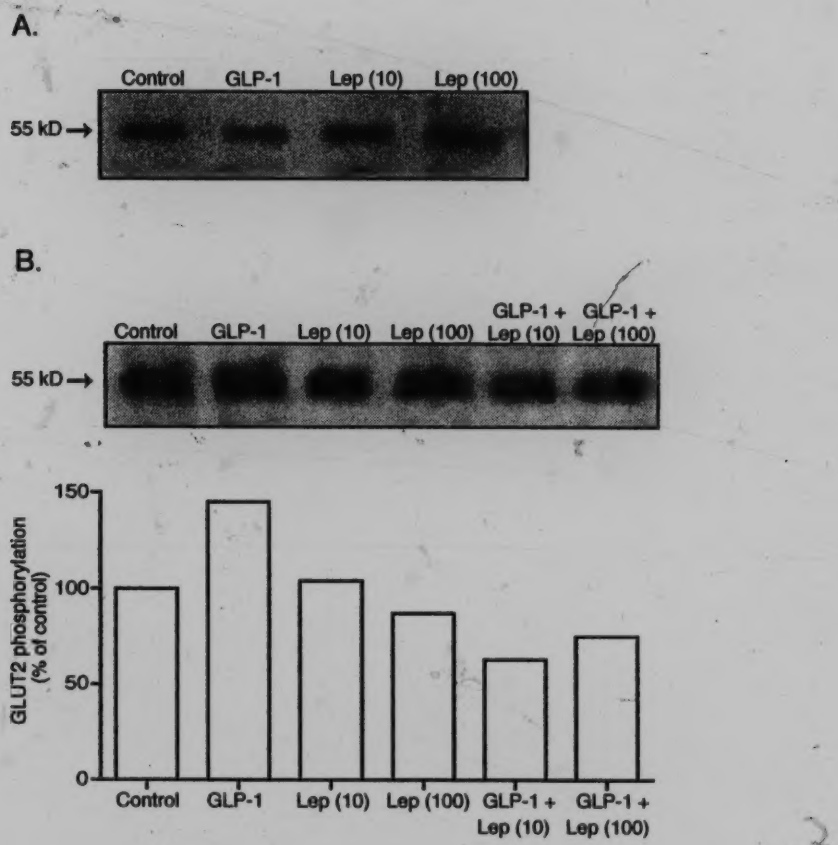

Figure 2.4. Transporter regulation by leptin and GLP-1. A. Effects of GLP-1 and leptin on GLUT2 protein expression in INS-1 cells. Cells were serum starved for 2 h prior to treatment with 10 or 100 ng/ml leptin for 30 min or 10 nM GLP-1 treatment for 15 min. Membrane proteins were gel-fractionated and immunoblotted with GLUT2 antiserum. B. Effects of GLP-1 and leptin on GLUT2 phosphorylation in INS-1 cells. Cells labeled with ^{32}P were treated with 10 nM GLP-1 and 10 or 100 ng/ml leptin for 30 min, or co-treated with 10 or 100 ng/ml leptin (30 min) with 10 nM GLP-1 (15 min). Cells were lysed, harvested and immunoprecipitated with GLUT2 antibody. After samples were separated by 10% SDS-PAGE and transferred to nitrocellulose, ^{32}P labeling was analyzed on a phosphimager, and densitometric values were determined. Values reported are mean densitometric values from two experiments.

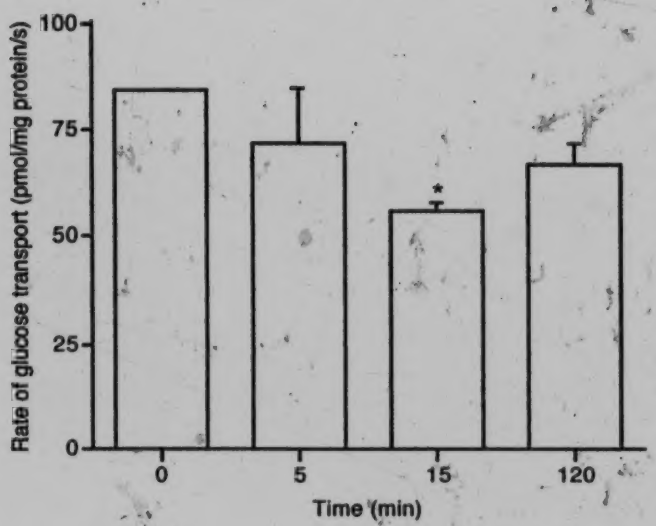

Figure 2.5. Effects of leptin on glucose transport in isolated hepatocytes. Prior to exposure to ^{3}H-3-O-MG, hepatocytes were treated with 10 ng/ml leptin for various periods. Values are presented as mean + SEM. *P<0.05, n=4.

CHAPTER 3

LEPTIN INCREASES HEPATIC INSULIN SENSITIVITY AND PROTEIN TYROSINE PHOSPHATASE 1B EXPRESSION

3.1. Introduction

The adipocyte-derived hormone leptin plays a key role in body mass regulation and feeding behavior (1). Rodents that fail to produce leptin (*ob/ob* mice) or are resistant to leptin (*db/db* mice and *fa/fa* rats) develop obesity and insulin resistance similar to the metabolic abnormalities observed in humans with type 2 diabetes. Exogenous administration of leptin to *ob/ob* mice can dramatically normalize the diabetic phenotype, reversing the hyperglycemia, hyperinsulinemia, hyperlipidemia, and improving insulin sensitivity (2-4). Because leptin interacts with receptors in the hypothalamus to suppress appetite (5), the reduction in plasma insulin and glucose may result, in part, from the inhibition of food intake and subsequent weight loss. However, the detection of both long (ObRb) and short isoforms (ObRa, -Rc, -Rd, -Rf) of the leptin receptor in insulin-targeted tissues such as muscle, adipose and liver (6, 7) provides evidence for peripheral leptin actions that may contribute to the regulation of insulin sensitivity. Indeed, whereas the mechanism by which leptin improves insulin sensitivity remains unknown, a number of studies have demonstrated the interaction between leptin and intermediates in the insulin signalling cascade including the insulin receptor (IR) (8), insulin receptor substrates (IRS) (8-10), phosphatidylinositol 3-kinase (PI3K) (9-11), protein kinase B (PKB) (9, 11) and phosphodiesterase (PDE3B) (11, 12).

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Because early steps in the insulin signalling cascade involve phosphorylation of tyrosyl residues on cellular proteins, the efficiency of insulin action depends on the balance between the competing action of a spectrum of protein tyrosine kinases and protein tyrosine phosphatases (PTPs). PTPs such as leukocyte common antigen-related phosphatase (LAR), Src homology 2-containing phosphatase 2 (SHP2), receptor protein tyrosine phosphatase-α, and protein tyrosine phosphatase 1B (PTP1B) have been identified in all the major target tissues of insulin, including muscle, liver and adipose tissue (13). Not all PTPs appear to be negative regulators of insulin signalling. For example, SHP2 acts to positively regulate components of the insulin signalling cascade in Chinese hamster ovary (CHO) cells (14) and in *Xenopus oocytes* (15). Although PTPs have potential for being important modulators of insulin sensitivity, the correlation between PTPs and the insulin resistant state, *in vivo,* is controversial. Despite reports of increased PTP activity in adipose tissue (16) and muscle (17, 18) from obese insulin-resistant human subjects, other studies have described a reduction of PTP activity in tissues of rodent models of insulin resistance (19) and type 2 diabetic patients (20-23). Due to these discrepancies, the roles of PTPs in normal physiology and the pathophysiology of insulin resistance and diabetes remain unclear.

One specific PTP, PTP1B, has been implicated as an important negative regulator of insulin signalling (24-29). PTP1B antisense oligonucleotide treatment of *ob/ob* and *db/db* mice resulted in increased phosphorylation of IR, IRS-1 and IRS-2 in liver tissue. Furthermore, PTP1B knock-out mice show improved insulin sensitivity with notably enhanced insulin-induced phosphorylation of IR and IRS-1 in liver and muscle and glucose uptake in muscle (28, 29). Therefore, down-regulation of PTP1B is a potential mechanism by which leptin could improve insulin sensitivity. We explored this possibility in *ob/ob* mice and somewhat surprisingly, we found that leptin acutely increased insulin sensitivity in liver, despite increased expression of PTP1B.

3.2. Materials and Methods

Administration of leptin in vivo

Male *ob/ob* mice on the Aston University background (30) were maintained by the Department of Agricultural, Food and Nutritional Sciences, University of Alberta (Edmonton, Alberta, Canada) and housed individually with a 12 h light-dark cycle. Animal studies were conducted under the approval of the Health Sciences Animal Policy and Welfare Committee, University of Alberta. Mice (~20 wk old) were administered, by IP injections, either PBS or 0.5 μg/g recombinant murine leptin (PeproTech Inc., Rocky Hill, NJ), twice daily for 2 days or 2 weeks. Body weight, chow consumption, plasma glucose and insulin were measured daily. For the 2 day cohort, after the 2 days of leptin treatment, mice were fasted for 4h following the final leptin dose, then subjected to either an IP insulin tolerance test (ITT) or an oral glucose tolerance test (OGTT). For the ITT, mice received an IP injection of 50 U/kg of human insulin (Novo Nordisk A/S, Bagsværd, Denmark). For the OGTT, mice were given 1.5 mg/g glucose as a 50% solution by gavage. Tail vein blood was collected at time 0 and at 10, 20, 30, 60, 90, and 120 min after the insulin injection for the ITT or the glucose bolus for the OGTT. Samples were centrifuged at 18300 x g in a microcentrifuge for 5 min at 4°C. Serum was separated and stored at -20°C for later analysis. Plasma glucose was determined using a calorimetric enzymatic assay kit (Sigma, St. Louis, MO), scaled down for use in 96-well microtiter plates. The area under the curve for the OGTT above the value at time 0 was calculated with Prism 4 Software by applying the trapezoidal rule to incremental changes (GraphPad Software Inc., San Diego, CA). Plasma insulin concentrations were measured using an ultrasensitive mouse insulin EIA kit (ALPCO Diagnostics, Windham, NH). Approximately 30 min after the OGTT, mice were anesthetized with 50 mg/kg ketamine hydrochloride/xylazine (Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada/Bayer Inc., Toronto, ON, Canada), a laparotamy was performed and 25 mU/kg human insulin was infused in the portal vein using an ultrafine syringe and needle (28 G). Two min after the insulin infusion, the

epididymal fat pads, soleus, plantaris, and gastrocnemius muscles and liver samples from the mice were excised and snap frozen. Protein extracts from tissues samples were obtained by resuspending tissue samples in lysis buffer (50 mM Hepes, 1% Triton-X, 2 mM EDTA, 200 mM NaF, 10 mM $Na_2P_4O_7$, 1 mM phenylmethylsulfonylfluoride, 1 mM Na_3VO_4, 10 μg/ml protease inhibitor cocktail, all reagents from Sigma-Aldrich Canada Inc., Oakville, ON, Canada) in lysing matrix D FastRNA tubes (Q-BIOgene Inc., Carlsbad, CA). The 2 wk cohort of mice were administered leptin similarly to the 2 day cohort. However the 2 wk cohort did not undergo ITT or OGTT but were monitored for 2 weeks. Six hours following the last leptin dose, a laparotomy was performed under anesthesia and liver tissue was extracted. Tissues were homogenized on a FastPrep FP120 (Q-BIOgene Inc.) and extracts were centrifuged at 18300 x g for 15 min to separate insoluble matter. The supernatant was removed for determination of protein concentration (DC protein assay, Bio-Rad Laboratories Canada Ltd., Mississauga, ON, Canada), and then used for further analysis.

Treatment of HepG2 cells for anti-phosphotyrosine immunoblotting of IR

HepG2 cells were obtained from American Type Culture Collection (Manassas, VA). Cells were cultured in 5 mM glucose at 37°C, 5% CO_2/95% air in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all reagents from Life Technologies Inc., Grand Island, NY). Cells were grown to approximately 70% confluency, then cultured for ~16 h in serum-free DMEM. After the 16 h starvation period, cells were treated with 100 ng/ml recombinant human leptin (PeproTech Inc.) for 1-12 h in serum-free DMEM. After the leptin incubation period, medium was removed and replaced with medium containing 100 nmol/L insulin for 2 min. At the end of the treatment period, cells were washed twice in ice-cold Dulbecco's PBS (D-PBS), then lysed in lysis buffer, harvested and measured for protein concentration, then stored at -70°C until further analysis. For analysis of tyrosine phosphorylation of the β subunit of IR

(IRβ), 2 mg protein lysates from tissues and HepG2 cells were resuspended in 400 μL lysis buffer and immunoprecipitated by incubation at 4°C overnight with an antibody to IRβ (Santa-Cruz Biotechnology, Inc., Santa Cruz, CA) and protein A conjugated protein A/G (Pierce Chemical Company, Rockford, IL). Immunoprecipitates were washed 3 times with lysis buffer and then boiled in sample buffer (1 M TrisCl, pH 6.8, 30% v/v glycerol, 5% v/v SDS, 0.1% wt/v bromophenol blue, 5% v/v β-mercaptoethanol). The resulting supernatants were separated on an 8% SDS-PAGE gel and transferred overnight at 4°C to nitrocellulose membrane (Osmonics Inc., Westborough, MA). The membranes were blocked in TBS buffer with Tween-20 (20 mM Tris, 150 mM NaCl, 0.1% Tween-20) containing 5% bovine serum albumin (all reagents from Sigma-Aldrich Canada Inc.). Immunoblotting was performed using a monoclonal mouse anti-phosphotyrosine antibody (PY99; sc-7020, Santa Cruz Biotechnology, Inc.). Membranes were washed and incubated with a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP)(sc-2005, Santa Cruz Biotechnology, Inc.). Protein content was visualized by enhanced chemiluminescence (ECL detection kit, Amersham Pharmacia Biotech, Uppsala, Sweden) and densitometric values were determined with Molecular Dynamics ImageQuant software (Amersham Pharmacia Biotech). IR and IRS-1 protein amounts were normalized by re-blotting the membrane with rabbit-polyclonal antibodies to the carboxy terminus of IRβ chain of human origin (sc-711, Santa Cruz Biotechnology, Inc.) and carboxy terminus of IRS-1 of human origin (sc-559, Santa Cruz Biotechnology, Inc.) after 'stripping' the membrane in buffer containing 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7 at 50°C for 30 min. Membranes were washed and incubated with a donkey antirabbit secondary antibody conjugated to HRP (NA934, Amersham Pharmacia Biotech). Protein content was visualized as described above.

Immunoblotting of PTPs

Protein lysates (20 μg) from tissue and HepG2 samples were boiled in sample buffer, then run on 10% SDS-PAGE gel and transferred overnight at 4°C to nitrocellulose membrane. Immunoblotting was performed as described above using either a goat polyclonal antibody to PTP1B of human origin (sc-1718, Santa Cruz Biotechnology, Inc), mouse monoclonal antibody to PTP1B of human origin (PH01, Oncogene Research Products, Boston, MA), rabbit polyclonal antibody to SHP2 of human origin (sc-424, Santa Cruz Biotechnology, Inc.), and goat polyclonal antibody to LAR of rat origin (sc-1119, Santa Cruz Biotechnology, Inc). Secondary antibodies used were HRP-conjugated, rabbit anti-goat (AP106P, Chemicon International Inc., Temecula, CA), goat anti-mouse, and donkey anti-rabbit respectively.

In vitro and in vivo visualization of Cy3-leptin

Murine leptin (PeproTech Inc.) was coupled to iodocarbocyanine (Cy3) monofunctional reactive dye (Amersham Biosciences UK Ltd) in 0.1 M bicarbonate buffer, pH 9.2, and purified on an Econo-Pac 10DG column (Bio-Rad Laboratories, Richmond, CA) in PBS (31). For the *in vitro* studies, Chinese hamster cells expressing ObRb (CHO-ObRb) cells were a kind gift from Dr. Takashi Murakami, Tokushima, Japan (32). Cells were cultured at 37°C, 5% CO_2/95% air in Ham's F-12K nutrient mixture with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate, supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (Life Technologies Inc.). For the experiment, cells were seeded onto Lab-Tek chamber slides (Nalge-Nunc Intl, Rochester, NY) in F-12 medium supplemented with 10% FBS. After overnight culture, slides were moved to 4°C, washed with ice-cold PBS and incubated at 4°C for 1 h with ~40 nmol/L Cy3-leptin in PBS. After several rinses with ice-cold PBS, cells were fixed for 10 min with ice-cold 4% paraformaldehyde, rinsed again, and coverslipped. Cells were examined on Leica DMIRB microscope (Leica Microsystems, Germany) and fluorescence was viewed under a Cy3 filter.

Images were obtained with a Photometrics CoolSNAP camera (Roper Scientific Inc., Trenton, NJ) and IPLab Spectrum analysis software (Signal Analytics, Vienna, VA). For the *in vivo* studies, C57Bl6 mice (~20 wk old, Jackson Laboratory, Bar Harbor, ME) were injected with 100 ug of Cy3-leptin or uncoupled Cy3 (control), via tail vein injection under anesthesia. After 5 min, a laparotomy was performed and mice were perfused via the portal vein with cold PBS (5 mL/min) for 1 min, then cold 4% paraformaldehyde in PBS, pH 7.4 (5 ml/min) for 2 min with a multi-speed infusion pump (Harvard Apparatus Co., Millis, MA). Liver tissues were removed immediately after perfusion and stored in cold 4% paraformaldehyde in PBS and kept shielded from light until they were prepared for microscopic examination. Tissues were embedded in Tissue-Tek OCT compound (IMEB Inc., San Marcos, CA) and frozen, then and cut into 10 μm cryosections on a cryostat, Cryocut1800 (Reichert-Jung, Germany). Specimens were mounted with Fluoromount antifade medium (Electron Microscopy Sciences, Fort Washington, PA) and stored at 4°C until examined. Tissue sections were examined and images were obtained as described above.

Treatment of CHO-ObRb and FAO for STAT3 phosphorylation

CHO-ObRb cells and FAO hepatoma cells (kind gift from Dr. C. Ronald Kahn, Boston, MA) were cultured in 5 mmol/L glucose at 37°C, 5% CO_2/95% air in F-12 and RPMI medium, respectively, supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (all reagents from Life Technologies Inc.). Cells were grown to approximately 70% confluency, then cultured for ~4 h in serum-free medium. Cells were treated with 100 ng/ml recombinant human leptin (PeproTech Inc.) for 15 min in serum-free medium. Following the leptin incubation period, cells were washed twice in ice-cold D-PBS, then lysed in lysis buffer, harvested and measured for protein concentration, then stored at -70°C for further analysis.

For analysis of tyrosine phosphorylation of STAT3, 750 μg protein lysates were resuspended in 400 μL lysis buffer and immunoprecipitated by incubation at

4°C overnight with an antibody to STAT3 (sc-482, Santa-Cruz Biotechnology, Inc.) and protein A conjugated protein A/G (Pierce Chemical Company, Rockford, IL). Immunoprecipitates were washed 3 times with lysis buffer and then boiled in sample buffer. The resulting supernatants were separated on a 10% SDS-PAGE gel and transferred overnight at 4°C to nitrocellulose membrane (Osmonics Inc., Westborough, MA). Immunoblotting was performed, as described above, using a monoclonal mouse anti-phosphotyrosine antibody (PY99; sc-7020, Santa Cruz Biotechnology, Inc.). STAT3 protein expression was determined by reblotting the membrane with a rabbit-polyclonal antibody to the carboxy terminus of STAT3 of mouse origin (sc-482, Santa Cruz Biotechnology, Inc.). Detection of signal was detected as described above.

Immunocytochemical detection of STAT3 in CHO-ObRb, FAO and ob/ob hepatocytes

CHO-ObRb and FAO cells were cultured onto glass coverslips in 10% FBS supplemented F-12 and RPMI media, respectively. When cells reached a confluency of ~70%, media was aspirated and cells were incubated in serum-free media for 4 h prior to receiving any hormone treatment. Hepatocytes were isolated by collagenase perfusion of the livers of *ob/ob* mice fed *ad libitum*, as previously described (33), with modifications. In brief, the hepatic portal vein was cannulated and perfused with a Ca^{2+}-free solution, pH 7.4 (142 mmol/L NaCl, 6.7 mmol/L KCl, 10.1 mmol/L Hepes, 5.5 mmol/L NaOH), prior to perfusion with Type IV collagenase, pH 7.6, (Sigma-Aldrich Canada Inc., Oakville, ON, Canada). Cells were isolated, trypan blue (Sigma-Aldrich) stained for viability, and cultured on collagenase-coated glass coverslips in DMEM medium, pH 7.4, (Life Technologies Inc.) supplemented with 10% FBS for ~1 h at which time cells had adhered to the slides. Medium was then changed to serum-free DMEM for 4 h at which point medium was aspirated and cells were incubated in DMEM containing leptin (100 ng/ml) at 37°C. CHO-ObRb and FAO cells were similarly treated in their respective media. After 30 min of

incubation, cells were washed with ice-cold PBS and then fixed in methanol-acetone for 15 min. All subsequent steps were performed at RT. Cells were washed with PBS, permeabilized with PBS containing 0.1% tween at for 30 min, then blocked with 3% BSA in PBS at for 30 min. Cells were incubated with rabbit polyclonal STAT3 antibody (sc-482, Santa Cruz Biotechnology Inc.) for 1 h, washed with PBS, then incubated with Cy3-conjugated AffiniPure Donkey anti-rabbit IgG (Jackson ImmunoResearch Laboratories, Inc.) for 45 min. After a final wash with PBS, coverslips were mounted onto glass microscope slides with fluoromount. Cy3 fluorescence was visualized as described above.

Construction and propagation of recombinant adenovirus

The recombinant adenovirus containing PTP1B cDNA was generated as previously described (34, 35). The cDNA encoding either β-galactosidase (β-gal) or PTP1B was subcloned into pACCMV.PLPASR(+) plasmid. This plasmid contains 1.3 map units of adenovirus 5 (Ad5) left end, cytomegalovirus early promoter, PUC19 polylinker site and SV40 poly A signal sequences, followed by map units 9-18 of the Ad5 genome. The resulting recombinant plasmid was then co-transfected into HEK293 packaging cells with pJM17 plasmid, which carries Ad5 genomic DNA. Mature recombinant Ad5 encoding β-gal or PTP1B were generated after *in vivo* homologous recombination between these two plasmids. For propagation of recombinant Ad5, HER911 cells were transfected with the recombinant viruses. When a complete cytopathic effect was observed (about 36-48 h later), cells were harvested by gentle scraping into culture media followed by five freeze/thaw cycles to break the cells. The lysates were cleared by centrifugation at 5000 rpm for 10 min. The cell lysates were mixed well with 3 g of cesium chloride (densitiy: top 1.3 g/ml CsCl, bottom 1.4 mg/ml). After centrifugation at 33000 rm for 1 h at 20°C, virus bands were carefully aspirated from the interface and transferred to sterile tubes. The viruses were then purified by two passages through Sepharose CL-4B spin columns. Glycerol was added in a final concentration of 10% and viruses were stored at -80°C. Virus titers were

determined by plaque assay. Briefly, 90% confluent HER911 cells (grown on 150 mm plates) were infected with serial dilutions of the virus stock. The infected cell monolayer was then overlaid with agarose in minimal essential medium (MEM)(LifeTechnologies Inc.). Plates were incubated at 37°C until visible plaque colonies were observed (6 to 9 days). The number of visible plaque colonies was counted to determine virus titers.

PTP1B overexpression in HepG2 cells

HepG2 cells were grown to about 70% confluency on 60 mm plates. Culture medium was then replaced with 0.5 mL of D-PBS with 4.3 mmol/L calcium (D-PBS-Ca^{2+}) containing 6.7×10^9 plaque forming units (PFU) of adenoviruses expressing either β-gal (Adβ-gal) or PTP1B (AdPTP1B), for 30 min. Fresh culture media was then added to the plates and cells were returned to CO_2 incubator at 37°C. Thirty-six hours following infection, cells were washed and incubated in serum-free DMEM for 6 h. Serum-starved cells were then treated with D-PBS or insulin (100 nmol/L) for 2 min, after which cells were washed twice with ice-cold D-PBS, snap frozen in liquid N_2 and lysed in cold lysis buffer. Measurement of IR phosphorylation and PTP1B protein expression were perfomed as described above.

Statistical analysis

Group differences were evaluated by ANOVA analysis and Tukey post-hoc tests with Statview software (SAS Institutes Inc., Cary, NC), with $P<0.05$ deemed as significant.

3.3. Results

Effects of leptin on body weight and food consumption in ob/ob mice

Before leptin treatment, *ob/ob* mice had body weights that were ~2 fold higher than the body weights of age-matched lean control mice (Table 1). After 2 days of treatment, body weights of mice in all four treatment groups did not change significantly. Therefore, as previously demonstrated (36), a treatment period of 2 days of leptin (0.5 μg/g, twice daily) was insufficient to reduce body weight in *ob/ob* mice. PBS-treated *ob/ob* mice showed no significant difference in food consumption after 2 days of treatment. In comparison, leptin-treated *ob/ob* mice consumed slightly less food after 1 day of treatment, which did not reach statistical significance (P=0.12, day 1 vs 0). After 2 days of leptin treatment, food intake was significantly reduced in the *ob/ob* mice (P<0.01, day 2 vs 0) but when compared to the food consumed by PBS-treated *ob/ob* mice on day 2, the food intake by leptin-treated mice was not significantly different (P=0.07). Leptin treatment similarly reduced food consumption in +/+ mice after 2 days (P<0.05, day 2 vs 0).

Effects of leptin on serum glucose and insulin in ob/ob mice.

Consistent with a diabetic phenotype, *ob/ob* mice had elevated levels of circulating insulin (~30 fold) and glucose (~2 fold) compared to their lean counterparts (Table 1). After day 1, leptin-treated *ob/ob* mice exhibited ~40% reduction in plasma glucose. Following the second day, glucose levels were essentially normalized by leptin in the *ob/ob* mice to levels observed in the lean mice. In comparison, PBS treatment did not reduce plasma glucose concentrations in *ob/ob* mice. Whereas insulin levels in *ob/ob* mice treated with PBS remained elevated over the 2 days of treatment, leptin dramatically reduced plasma insulin levels in *ob/ob* mice by ~55% after 1 day and by ~75% after day 2 (Table 1). Leptin treatment of +/+ mice had no significant effect on either plasma glucose or insulin levels.

Effect of leptin on whole-body insulin sensitivity

Insulin sensitivity in mice was assessed by ITT. Upon injection of insulin, plasma glucose levels were significantly reduced to 50% of fasting glucose levels by 20 min in leptin-treated *ob/ob* mice whereas this effect was not attained until 90 min after the insulin challenge in the PBS-treated *ob/ob* mice (Fig. 3.1A). Glucose levels were significantly different between the two groups by 20 min ($P<0.001$) and remained different at 120 min post insulin. While glucose levels in *ob/ob* mice receiving PBS reached the nadir at 50% of fasting levels, glucose levels were reduced to 23% of fasting levels in leptin-treated *ob/ob* mice, indicating improved insulin sensitivity with leptin treatment.

Effect of leptin on oral glucose tolerance

After 2 days of treatment, all groups were subjected to an OGTT to assess differences in glucose homeostasis. Plasma glucose concentrations before the oral glucose load were reduced in the *ob/ob* leptin (11.2 ± 1.2 mM) compared with the *ob/ob* PBS mice (18.5 ± 2.4 mM, $P<0.01$, *ob/ob* leptin vs *ob/ob* PBS) and was not significantly different from lean PBS mice (9.0 ± 0.4 mM, $P=0.30$, *ob/ob* leptin vs +/+ PBS, time 0)(Fig. 3.1B). However, in response to a glucose challenge, both the *ob/ob* leptin and *ob/ob* control groups had similar area under the glucose curve values (AUC above the value at time 0; 1220 ± 104 vs 1074 ± 153 mM•min, *ob/ob* leptin vs *ob/ob* PBS, $P=0.36$)(Fig. 3.1C). Leptin had little effect on basal glucose concentrations (9.8 ± 0.4 vs 9.0 ± 0.4 mM, +/+ leptin vs +/+ PBS, $P=0.26$), or AUC in lean mice (AUC; 223 ± 28 vs 286 ± 24 mM•min, +/+ leptin vs +/+ PBS, $P=0.13$). The finding that 2 days of leptin reduces fasting plasma glucose concentrations but has no effect on glucose disposal in *ob/ob* mice suggests that leptin may specifically target the liver and the regulation of hepatic glucose production in the basal state.

Leptin receptor expression and signalling in transfected CHO cells and hepatocytes

To determine if the *in vivo* effects of leptin in *ob/ob* mice could be due to direct action in the liver, we first evaluated whether leptin receptors are present on hepatocytes in these animals. Iodocarbocyanine-coupled leptin (Cy3-leptin) was generated and tested for binding ability to leptin receptors following incubation with untransfected CHO cells and CHO cells stably transfected with the ObR gene (CHO-ObR). Although no fluorescence was detected in untransfected CHO cells exposed to Cy3-leptin, distinct membrane localization of Cy3-leptin was visualized in CHO-ObR cells (Fig. 3.2A). Cy3-leptin was administered via tail-vein to C57Bl6 mice for the extraction of tissue to assess the distribution of leptin receptor binding in liver. In mice that received uncoupled Cy3, fluorescence was minimal and non-specific. However, Cy3-leptin was distributed with distinct localization to hepatocyte cellular membranes (Fig. 3.2B). We next looked at leptin's capability of signalling in hepatocytes. It is believed that most of the physiological effects observed with leptin is through its signalling via ObRb. The 304 amino acid intracellular domain of ObRb contains putative motifs for JAK and STAT binding. Upon binding of JAK, the intracellular domain of the receptor becomes phosphorylated which then allows for binding, phosphorylation and activation of STAT proteins. Dimerization of STAT proteins causes their nuclear translocation, where they regulate gene transcription. CHO cells transfected with the ObRb gene (CHO-ObRb) and rat liver (FAO) hepatoma cells were treated with leptin and phosphorylation of STAT3 was evaluated. In both cell types, STAT3 phosphorylation was increased in a dose dependent manner (Fig. 3.2C). Further supporting the presence of functional leptin receptors in hepatocytes, nuclear STAT3 translocation upon leptin treatment was visualized in CHO-ObRb and FAO cells (Fig. 3.2D). Collectively, these observations provide support for the presence of functional leptin receptor signalling in liver cells.

Effect of leptin on IR and IRS-1 phosphorylation in tissues from ob/ob mice and in HepG2 cells

To evaluate whether the acute effects of leptin were tissue specific, we examined changes in IR phosphorylation of tissues harvested following an infusion of insulin (25 mU/kg) into the portal vein. To first determine whether insulin infused into the portal vein was detected at peripheral tissues, blood was collected from the tail vein of 4 h fasted lean mice to determine the circulating concentration of insulin at 0, 1, and 2 min after infusion. Prior to insulin infusion of 25 mU/kg, the basal insulin value was 0.236 ± 0.002 nM. After 1 min, insulin levels rose to 5.0 ± 0.4 nM, and remained elevated at 8.3 ± 1.5 nM after 2 min. IR immunoblots demonstrated insulin-induced IR phosphorylation in both liver and muscle tissues of lean controls (Fig. 3.3E). In liver and muscle, IR phosphorylation was reduced in *ob/ob* mice compared to wild-type lean mice consistent with insulin resistance in the *ob/ob* mice. Treatment with leptin (0.5 μg/g) twice daily for 2 days increased IR phosphorylation in liver but not skeletal muscle or adipose tissue (pTyr/IR densitometric ratios for *ob/ob* leptin vs *ob/ob* PBS: liver (1.26 vs 0.63, $P<0.01$), muscle (1.18 vs 0.95, $P=0.48$), adipose tissue (0.24 vs 0.21, $P=0.80$)) (Fig. 3.3). Downstream phosphorylation of hepatic insulin receptor substrate-1 (IRS-1) was similarly increased in leptin treated *ob/ob* mice (0.41 vs 0.82, $P<0.05$, *ob/ob* leptin vs *ob/ob* PBS)(Fig. 3.3B). Leptin had no effect on IR or IRS-1 phosphorylation on tissues from +/+ mice (data not shown). To determine whether the improvement in IR phosphorylation may have been a direct effect of leptin on hepatocytes, we treated human hepatoma cells, HepG2, with leptin (100 ng/ml) for various periods. IR phosphorylation appeared to increase within 3 h of leptin treatment (Fig. 3.4). Leptin thus appears to acutely improve insulin sensitivity in liver potentially via direct effects on hepatocytes.

Effect of leptin on PTP expression in tissues from ob/ob mice and in HepG2 cells

To determine whether PTPs may be involved in the effects of leptin on IR phosphorylation, we examined the protein expression levels of PTP1B in liver, skeletal muscle and adipose tissue. PBS-treated *ob/ob* mice had lower levels of PTP1B expression than lean mice in all tissues examined. Remarkably, leptin increased the expression of PTP1B in *ob/ob* mice to levels observed in +/+ mice, in liver, but not skeletal muscle or adipose tissue (Fig. 3.5A). The difference in PTP1B expression appeared to be a phosphatase specific event, since there were no differences in the expression of SHP2 or LAR in *ob/ob* mice treated with leptin compared to those treated with PBS (Fig. 3.5B). Liver tissue extracted from *ob/ob* mice treated with leptin over a period of two weeks also displayed increased PTP1B expression suggesting that leptin's effects after 2 days are retained even after 2 weeks (Fig. 3.5C). While leptin dramatically increased PTP1B expression in livers of *ob/ob* mice, it did not elevate PTP1B levels in +/+ mice (data not shown). To determine whether the effects of leptin on PTP1B may have been direct, HepG2 cells were treated with leptin for various periods and immunoblotted for PTP1B. Similar to its effects on IR phosphorylation, leptin increased PTP1B expression within 4 h of leptin treatment and reached a maximal value at 6 h (Fig. 3.6). Therefore, leptin-induced increases in PTP1B expression in liver of *ob/ob* mice after 2 days of treatment may be due to direct effects in hepatocytes.

Effect of PTP1B overexpression on IR phosphorylation in HepG2 cells

To clarify the role of PTP1B on insulin signalling in hepatocytes, PTP1B was overexpressed in HepG2 cells using a recombinant adenovirus expressing wild-type human PTP1B (AdPTP1B). Cells infected with AdPTP1B expressed PTP1B ~2.5 fold greater than cells infected with Adβ-gal (Fig. 3.7A). In control, non-transduced cells, insulin stimulation for 2 min increased IR phosphorylation by ~2 fold. Adβ-gal expression had no significant effect on basal or insulin-

stimulated IR phosphorylation. In PTP1B-overexpressing cells, basal and insulin-stimulated IR phosphorylation were reduced by ~50% (Fig. 3.7B).

3.4. Discussion

Changes in body weight, fat mass, and/or fat distribution similar to those associated with long-term leptin administration can independently alter insulin action, particularly in insulin-resistant and obese animal models (37, 38). To study the effects of leptin on glucose homeostasis in *ob/ob* mice that are independent of its effects on body weight, we examined changes in plasma glucose and insulin levels, and insulin sensitivity after two days of leptin administration. At this point, leptin had no effects on body weight but plasma glucose concentrations were reduced to levels observed in lean wild-type mice, and plasma insulin levels were significantly reduced in comparison to PBS-treated *ob/ob* mice. While food intake was normalized after 2 days of leptin treatment, several lines of evidence suggest that the effects of leptin on glucose homeostasis are largely independent of reduced food consumption (2, 39-42). In our study, after the first day of leptin administration, plasma glucose levels in the *ob/ob* mice were similar to the PBS-treated wild type mice, despite no significant reduction in food intake. Collectively, these results demonstrate that leptin can regulate glucose homeostasis independently of its effects on food intake and body weight.

The reduction in plasma glucose and concurrent decrease in plasma insulin indicate that insulin sensitivity was improved upon leptin treatment in the *ob/ob* mice. In support, insulin tolerance tests performed following the second day of treatment indicated that *ob/ob* mice were extremely resistant to the actions of insulin whereas leptin-treated *ob/ob* mice had significantly improved responses to insulin. Although leptin-treated mice appeared to be more insulin sensitive and basal glucose levels were normalized by leptin, oral glucose tolerance tests performed on the second day of treatment showed that glucose tolerance following an oral glucose challenge remained impaired. During the short treatment period, leptin was capable of affecting fasting but not post-prandial glucose homeostasis, which may reflect tissue specific leptin action and/or sensitivity. Indeed, whereas leptin markedly increased insulin-stimulated IR phosphorylation in liver after 2 days of treatment, insulin-induced IR

phosphorylation remained reduced in muscle and adipose tissue. *In vivo*, leptin has been reported to enhance insulin's action to inhibit hepatic glucose production (10, 43, 44). A reduction in high glucose production after leptin treatment is consistent with our observations. Although leptin action was observed in the liver, our data do not preclude the possibility that leptin may similarly have affected skeletal muscle and adipose tissue after a longer treatment period or with a higher dose of leptin.

Our observations of leptin binding sites on hepatocytes, leptin-induced translocation of STAT3 and increased insulin-stimulated IR phosphorylation suggests that leptin mediated changes in glucose homeostasis may in part be due to direct actions of leptin on hepatocytes. This does not exclude the possibility that the *in vivo* effects observed in the liver are mediated centrally (44-48) or via other tissues. Because leptin curtails insulin secretion from pancreatic β cells (49), it is possible that the increase in hepatic IR phosphorylation after leptin treatment resulted indirectly from the reduction in plasma insulin levels and subsequent improvements in insulin sensitivity. However, in agreement with our *in vitro* studies, leptin has been reported to stimulate JAK2 phosphorylation in liver (50) and interact directly with the insulin signalling cascade, both augmenting and antagonizing insulin signalling in hepatocytes (8, 9, 51).

Mice lacking the PTP1B gene exhibit significantly enhanced whole body insulin sensitivity and are protected from diet-induced obesity (28, 29). The improvement in insulin sensitivity is correlated with enhanced and/or sustained tyrosyl phosphorylation of IR and IRS-1 in response to insulin stimulation in liver and muscle but not in adipose tissue (28). Based on these studies, we hypothesized that leptin improved insulin sensitivity by reducing PTP1B expression. In liver, skeletal muscle and adipose tissue from *ob/ob* mice, we observed relatively low levels of PTP1B protein expression, which appeared to be counterintuitive given the insulin-resistant state of these mice. However, this finding is consistent with the observations of reduced PTP1B protein expression in muscle biopsies from patients with type 2 diabetes in comparison to non-

diabetic control subjects (21, 22). Worm *et al.* hypothesized that the associated down-regulation of PTPase activity in type 2 diabetic patients was in attempt to compensate for the inability of the tissues to sense insulin (23). Alternatively, it has been proposed that although PTP1B levels are decreased, there may be a larger reduction in IR abundance. This would result in a higher ratio of PTP1B to IR in *ob/ob* in comparison to wild-type mice, thereby explaining the relative increase in insulin resistance in the *ob/ob* mice (25). In our study, however, we did not observe any difference in liver IR protein abundance between *ob/ob* and wild-type mice. This suggests that insulin resistance in *ob/ob* mice is unlikely to be attributed to overexpression of PTP1B.

Contrary to our original hypothesis that leptin could improve insulin sensitivity by reducing PTP1B expression, leptin treatment of *ob/ob* mice resulted in an increase in PTP1B protein expression in liver, the same tissue in which insulin sensitivity had evidently been restored. This effect of leptin appeared to be tissue-specific, as leptin did not increase PTP1B expression in skeletal muscle or adipose tissue. Furthermore, this effect of leptin may be mediated through direct effects on hepatocytes as leptin treatment of HepG2 cells also increased PTP1B protein levels. Although PTP1B expression may have been elevated in response to increased insulin signalling after leptin treatment *in vivo* (52), this does not account for our findings in the HepG2 cells that were treated with leptin in the absence of insulin. Therefore, leptin increases IR activation in the liver and can independently up-regulate PTP1B expression. Given the metabolic and growth-promoting functions of insulin (53, 54), the ability of PTP1B to counter-regulate the IR tyrosine kinase activity may be physiologically relevant. As insulin sensitivity is improved by leptin, a subsequent corresponding increase in PTP1B expression may serve to provide a "brake", and thereby intervene to attenuate undesirable insulin effects in cells. A negative-feedback loop is supported by the demonstration of PTP1B as a negative regulator of leptin signalling as well as insulin signalling (55, 56). This autoregulatory mechanism

might allow for more dynamic control over the downregulation of both insulin and leptin signalling.

In summary, our findings demonstrate that leptin is capable of improving whole body glucose homeostasis and increasing insulin sensitivity in liver of *ob/ob* mice independent of changes in body weight. The mechanism by which leptin enhances insulin sensitivity in this model remains to be elucidated, as it does not appear to involve a reduction of PTP1B protein levels. Instead, PTP1B expression is reduced in the insulin resistant state and is elevated when insulin sensitivity is restored. We propose that the increase in PTP1B in response to improved insulin sensitivity is part of a negative feedback loop designed to provide an autoregulatory means by which insulin can attenuate its own signalling cascade. Further understanding of the roles of leptin and PTP1B and their interaction with the insulin signalling pathway may provide additional insight into the regulation of insulin sensitivity and the pathogenesis of insulin resistance associated with obesity.

3.5. References

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Table 3.1. Effect of leptin administration *in vivo* on body weight, food intake, plasma glucose and insulin

			Leptin treatment period		
Parameter	**Group**	**n**	**Day 0**	**Day 1**	**Day 2**
Body weight	+/+ PBS	8	33.3 ± 0.6	32.6 ± 0.7	32.7 ± 0.7
(g)	+/+leptin	8	32.5 ± 0.5	31.3 ± 0.4	31.0 ± 0.4
	ob/ob PBS	13	56.5 ± 3.8	56.4 ± 3.7	56.4 ± 3.6
	ob/ob leptin	17	61.5 ± 4.0	60.6 ± 3.9	60.6 ± 4.0
Food intake	+/+ PBS	8	4.8 ± 0.3	4.1 ± 0.3	4.9 ± 0.6
(g/day)	+/+ leptin	8	4.6 ± 0.3	3.4 ± 0.2	3.1 ± 0.2*†
	ob/ob PBS	13	7.7 ± 0.6	7.4 ± 0.5	7.1 ± 0.5
	ob/ob leptin	17	8.0 ± 0.5	6.8 ± 0.6	5.7 ± 0.7*†
Plasma glucose	+/+ PBS	8	8.1 ± 0.3	9.5 ± 0.7	9.5 ± 0.5
(mmol/L)	+/+ leptin	8	8.1 ± 0.2	8.8 ± 0.5	9.7 ± 0.3
	ob/ob PBS	13	19.3 ± 2.0	18.4 ± 1.9	21.8 ± 2.1
	ob/ob leptin	13	16.8 ± 2.1	10.8 ± 0.8*†	11.3 ± 1.1*†
Plasma insulin	+/+ PBS	8	0.27 ± 0.03	0.31 ± 0.04	0.27 ± 0.04
(nmol/L)	+/+ leptin	8	0.30 ± 0.03	0.27 ± 0.03	0.24 ± 0.04
	ob/ob PBS	9	8.8 ± 3.3	7.8 ± 1.6	10.8 ± 2.6
	ob/ob leptin	9	9.2 ± 1.6	3.9 ± 1.0	2.4 ± 0.8*†

Mice were treated with PBS or leptin (0.5 μg/g) twice daily for 2 days. Data are means ±SE. *$P \leq 0.05$ vs Day 0 of same treatment group, †$P \leq 0.05$ vs PBS on same day of treatment.

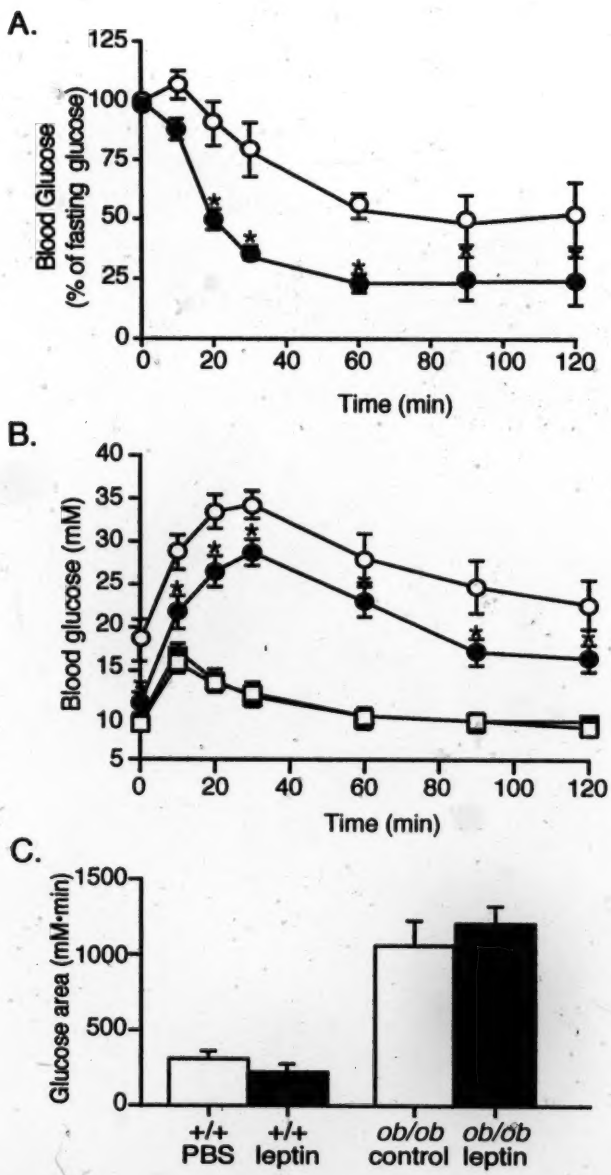

Figure 3.1. Leptin improves insulin sensitivity. *Ob/ob* and +/+ mice were treated with leptin or PBS for 2 d, after which mice were fasted for 4 h, then subjected to OGTTs or ITTs. A. ITTs in *ob/ob* mice treated with leptin (black circles, n=6) and *ob/ob* mice treated with PBS (white circles, n=6) plotted as mean ± SEM. B. plasma glucose profile after the administration of an OGTT plotted as mean ± SEM. Treatment groups were wild-type mice treated with PBS (white squares, n=17) or leptin (black squares, n=9) and *ob/ob* mice treated with PBS (white circles, n=9) or leptin (black circles, n=12). *, Difference in plasma glucose concentrations between leptin- and PBS-treated *ob/ob* mice at the same time point, $P<0.05$. C. The incremental area under the glucose curve between 0 and 120 min expressed as mean ± SEM.

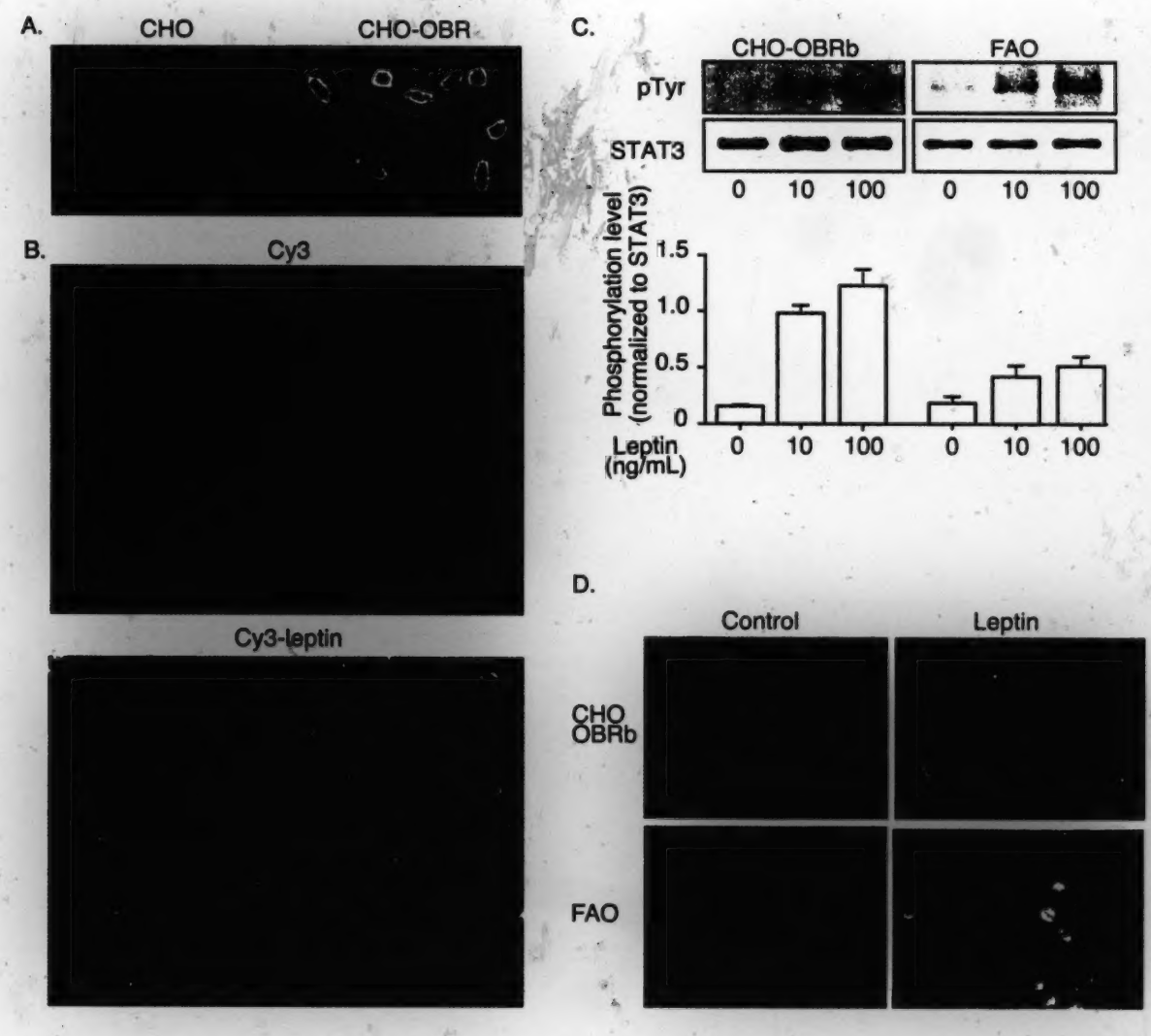

Figure 3.2. Leptin receptor immunoreactivity and signalling in hepatocytes. A. Detection of Cy3 fluorescence in CHO cells stably transfected with OBR gene and untransfected cells (control) that were incubated with Cy3-labelled leptin (4 nM) for 1 h at 4 C. B. Cy3 fluorescence in liver tissues extracted from C57BL6 mice 5 min after injection of 100 µl of Cy3-labelled leptin or Cy3 (control). C. Immunoblots of CHO-ObRb and FAO cell lysates that were immunoprecipitated with antibody to STAT3 and immunoblotted with antiphosphotyrosine antibodies. Membranes were stripped and reprobed with STAT3 antibody to normalize for protein loading. D. Immunofluorescent detection of STAT3 protein in CHO-ObRb and FAO cells after leptin treatment (100 ng/ml) for 30 min.

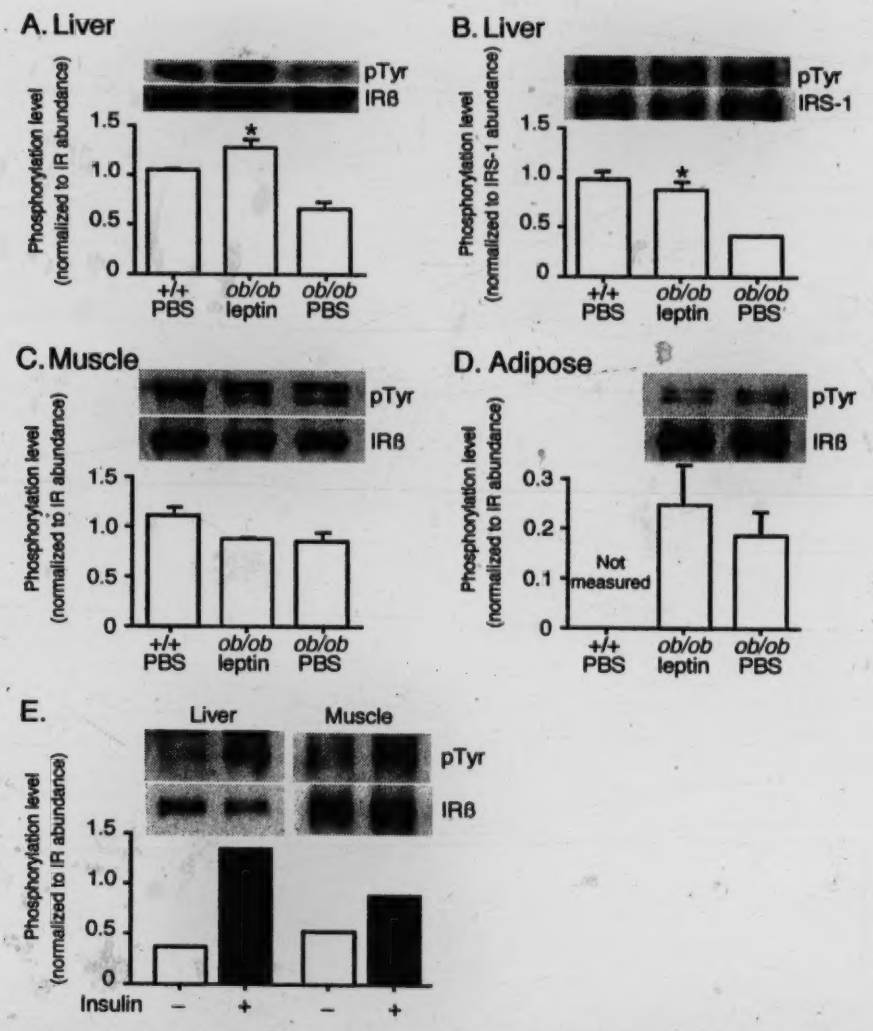

Figure 3.3. Leptin increases IR phosphorylation in a tissue-specific manner. After the final leptin or PBS treatment of day 2, mice were fasted for 4 h and then subjected to OGTT's. Approximately 30 min after the final OGTT time point, mice were given an infusion of insulin (25 mU/kg) via the portal vein. Two minutes after the insulin infusion, tissues were harvested, and protein lysates prepared from lean and *ob/ob* mice tissue extracts were immunoprecipitated with antibody to IR. Phosphorylation of IR was assessed by immunoblotting with antiphosphotyrosine antibody. Membranes were stripped and reprobed with IR antibody. Representative immunoblots depicting tyrosine phosphorylation of a 95 kDa IR β-subunit is shown for A. liver, C. Skeletal muscle, and D. adipose tissue. Immunoblots for IRS-1 in liver is shown in panel B. E. To determine whether insulin infusion via the hepatic portal vein was detected at peripheral tissues, IR phosphorylation was assessed in untreated lean mice that were fasted for 4 h before insulin infusion. Tissues were extracted 2 min after infusion. Below each immunoblot is displayed the intensities (arbitrary units) of phosphotyrosine expression, corrected for total IR or IRS-1 (n=3, *P<0.05).

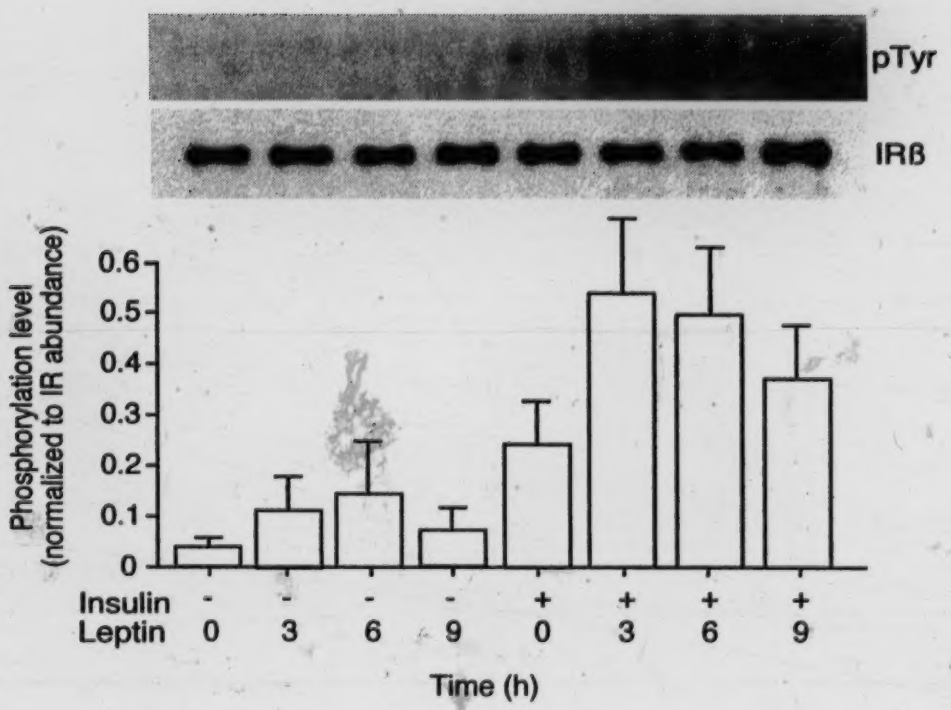

Figure 3.4. Leptin increases IR phosphorylation in HepG2 cells. HepG2 cells were serum starved overnight before 100 ng/ml leptin treatment (0-9 h). After the leptin incubation period, media were removed and replaced with media containing 100 nM insulin for 2 min. Cellular protein lysates prepared after treatment were immunoprecipitated with an antibody to IR. Phosphorylation of IR was assessed by immunoblotting with antiphosphotyrosine antibody. Membranes were stripped and reprobed with IR antibody. Representative immunoblots depicting tyrosine phosphorylation of a 95-kDa IR β-subunit are shown. Below the immunoblots are displayed the mean intensities (arbitrary units) of phosphotyrosine expression, corrected for total IR ± SE (n=2)

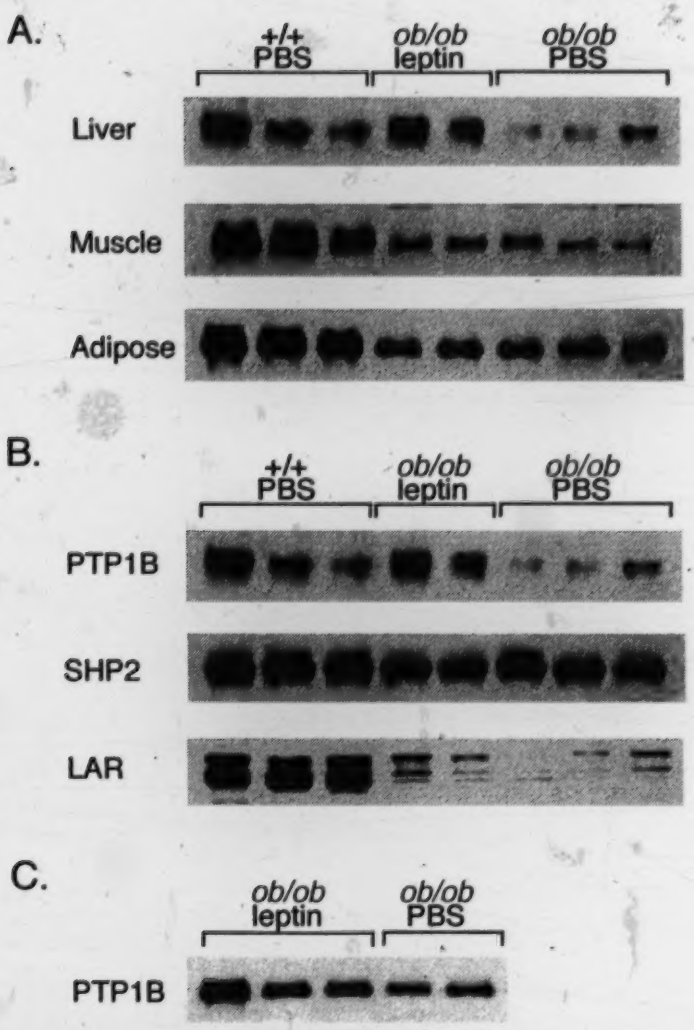

Figure 3.5. Leptin increases PTP1B expression in a tissue- and phosphatase-specific manner. Following the final leptin or PBS treatment on day 2, mice were fasted for 4 h and then subjected to OGTTs. Approximately 30 min after the OGTT, mice were given an infusion of insulin (25 mU/kg) via the hepatic portal vein. Two minutes after the insulin infusion, +/+ and *ob/ob* mice tissues were harvested and protein lysates prepared were immunoblotted with antibodies to either PTP1B or SHP2.
A. Immunoblots showing PTP1B (50 kDa) protein expression in liver, skeletal muscle, and adipose tissue extracted from mice treated for 2 d with leptin or PBS.
B. Immunoblots of PTP-1B, SHP-2 (68 kDa) and LAR (212 kDa) protein expression in livers of mice treated for 2 days with leptin or PBS. C. Immunoblots showing PTP1B (50 kDa) protein expression in liver extracted from mice treated for 2 wk with leptin or PBS.

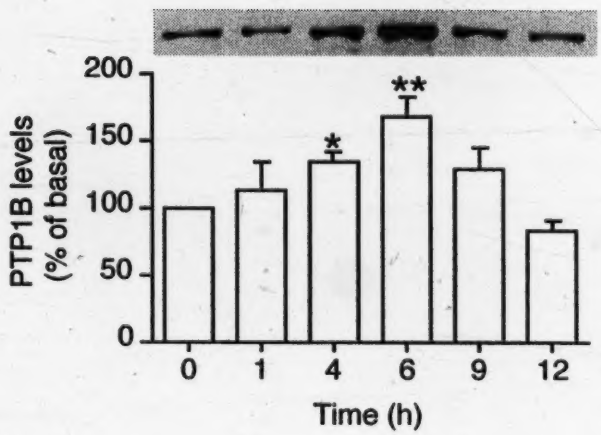

Figure 3.6. Leptin increases PTP1B protein expression in HepG2 cells. HepG2 cells were serum starved overnight before 100 ng/ml leptin treatment (0-12 h). After the treatment period, cellular protein lysates were immunoblotted with anti-PTP 1B antibody. A representative immunoblot showing expression of a 50 kDa PTP1B protein is shown. Below the immunoblot is displayed the intensities, expressed as the percentage of PTP1B expression in PBs-treated HepG2 cells (n=4, *P<0.05, **P<0.01).

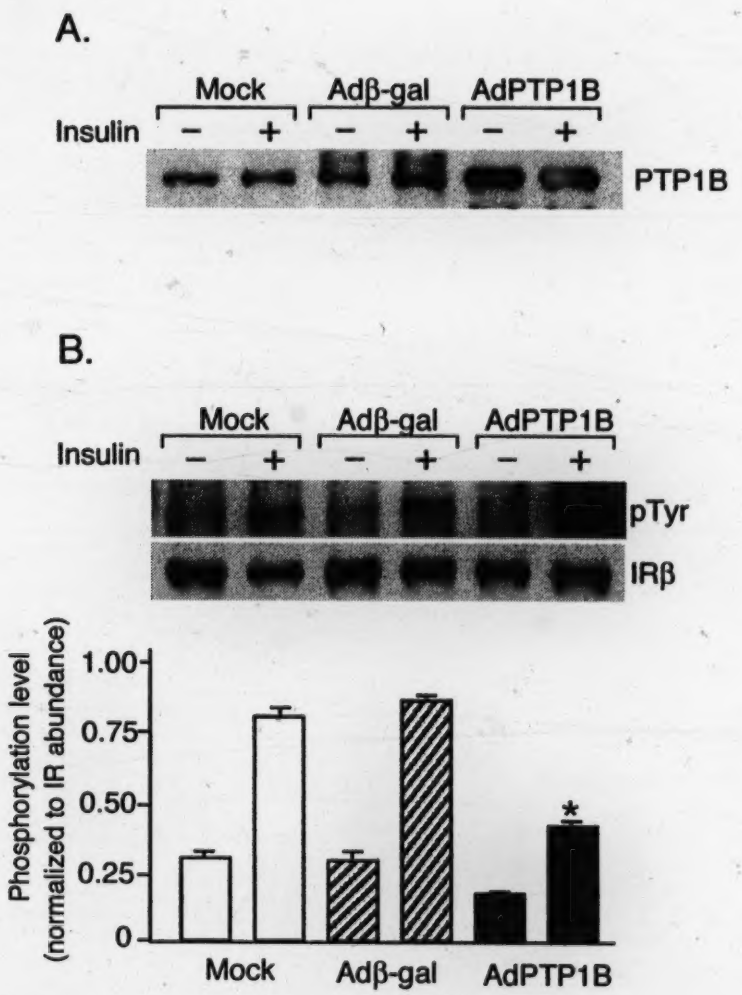

Figure 3.7. PTP1B overexpression in HepG2 cells decreases IR phosphorylation. HepG2 cells were treated either mock infected or infected with Adβ-gal or AdPTP1B. After 36 h, cells were serum starved for 6 h and then treated with insulin (100 nM) for 2 min before solubilizing. A. Immunoblot of cell lysates from HepG cells infected with Adβ-gal and AdPTP1B is shown. B. Immunoblot of tyrosine phosphorylation using an antiphosphotyrosine antibody is shown. Membranes were reprobed with IR antibody to determine the total amount of IR that was immunoprecipitated. Below the immunoblot is displayed the intensities (arbitrary units) of phosphotyrosine expression, corrected for total IR (white bars, mock infected: hatched bars, Adβ-gal infected; black bars, AdPTP1B infected; n=2, *P<0.001 comparison of different virally infected groups under same insulin-stimulatory condition).

CHAPTER 4

LEPTIN RESISTANCE FOLLOWING OVEREXPRESSION OF PROTEIN TYROSINE PHOSPHATASE 1B IN LIVER

4.1. Introduction

Hormone signalling typically involves an intricate balance between signalling activators and inhibitors. The stimulatory effects of protein tyrosine kinases and the inhibitory effects of protein tyrosine phosphatases largely define the actions of insulin. Appropriate insulin signalling minimizes large fluctuations in blood glucose concentrations and insures adequate delivery of glucose to cells. Therefore a signalling imbalance whereby the inhibitory actions of the phosphatases prevail causes insulin resistance, which if left untreated may lead to diabetes mellitus. Signalling of the adipocyte derived hormone leptin is similarly regulated. Leptin signalling begins with the phosphorylation and activation of janus kinase 2 (JAK2) and subsequently ends with the dephosphorylation and deactivation of JAK2. Leptin, an important satiety factor, functions both in hypothalamic satiety centers and peripherally as an accountant of caloric consumption and energy expenditure and is important in body weight regulation and the maintenance of normal glucose and fatty acid homeostasis (1-3). Resistance to leptin thus can lead to weight gain and elevated blood glucose levels. The pathophysiological mechanisms that cause leptin and insulin signalling pathways to become imbalanced such that obesity and diabetes develop have not been established.

Insulin signal transduction begins with insulin-induced autophosphorylation of tyrosine residues in the insulin receptor and subsequent recruitment of insulin receptor substrate (IRS) proteins, followed by

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activation of phosphophatidylinositol 3-kinase (PI3K) and downstream protein kinase B (PKB) ultimately leading to translocation of the glucose transporter GLUT4 to the cell surface membrane to facilitate glucose uptake. Protein tyrosine phosphatase 1B (PTP1B) attenuates insulin signalling by dephosphorylating the insulin receptor (IR) (4). Mice lacking the PTP1B gene ($PTP1B^{-/-}$) exhibit enhanced insulin sensitivity (5, 6), and treatment with PTP1B antisense oligonucleotides improves insulin sensitivity and normalizes blood glucose levels in diabetic mice (7, 8). Surprisingly, $PTP1B^{-/-}$ mice are also resistant to weight gain when given a high-fat diet (5, 6). This might be at least in part due the fact that these animals have enhanced sensitivity to leptin. JAK2 has been shown to be a substrate of PTP1B in cell lines (9, 10) and PTP1B negatively regulates leptin signalling in hypothalamic satiety centers by dephosphorylating JAK2 *in vivo* (11, 12). Given the dual role of PTP1B in attenuating leptin and insulin signalling, there is a great deal of interest in pursuing PTP1B inhibition to treat both obesity and diabetes (13, 14). However, the relative importance of PTP1B activity in regulating leptin signalling in peripheral tissues has not been established.

The observation that elimination of hypothalamic neurons expressing leptin receptors in $PTP1B^{-/-}$ mice has no effect on the insulin hypersensitivity of the animals and only partially reverses the protection from diet-induced obesity led Zabolotny *et al.* to conclude that PTP1B must also regulate additional body mass pathways, perhaps in the periphery (12). To explore the relevance of PTP1B in the liver to the actions of leptin, here we examine leptin function in animals in which hepatic PTP1B levels are elevated, either by high fat diet or with an adenoviral vector. Intriguingly, we discovered that elevated hepatic PTP1B expression results in a severe impairment of the ability of leptin to reduce both plasma glucose levels and food intake. These findings suggest that the liver is a significant locus for leptin actions on regulating both glucose homeostasis and food intake, and that PTP1B is an important negative regulator of these pathways and perhaps involved in the pathogenesis of diabetes associated with obesity.

4.2. Materials and Methods

Animals and diets

C57BL/6 mice (3 weeks old) were maintained by the University of Alberta Health Sciences Lab Animal Services and housed individually with a 12 h light-dark cycle. Animal studies were conducted under the approval of the Health Sciences Animal Policy and Welfare Committee, University of Alberta, Edmonton, AB, Canada. Following a one-week acclimatization period during which mice were provided ad libitum access to a purified diet containing 11% kcal as coconut oil (D12328, Research Diets, Inc., New Brunswick, NJ), mice were designated to be placed on low fat (LF) 11% diet or high fat (HF) 58% kcal coconut oil diet (D12330, Research Diets, Inc.). The composition of diet D12328 was 16.4% protein, 73.1% carbohydrate and 10.5% fat, whereas diet D12330 was 16.4% protein, 25.5% carbohydrate, and 58.0% fat. Caloric content for D12328 and D12330 is 4.07 and 5.56 kcal/g, respectively. Mice were maintained on these diets for 72 days and body weight and food consumption were measured daily. After 5 weeks on the diets, mice were fasted for 4 h and blood samples were collected for measurement of glucose, insulin and leptin levels. Plasma glucose was determined using a calorimetric enzymatic assay kit (Diagnostic Chemicals Ltd, Charlottetown, PEI, Canada), scaled down for use in 96-well microtiter plates. Plasma insulin and leptin concentrations were measured using ultrasensitive mouse insulin and leptin enzyme-linked immunoassay kits, respectively (American Laboratory Products Co., Windham, NH). After 72 days on the diets, mice were administered, by intraperitoneal (IP) injections, either phosphate buffered saline (PBS) or 2.5 μg/g recombinant murine leptin (PeproTech Inc., Rocky Hill, NJ), twice daily (at 0900 h and 1600 h) for 2 days. Body weight, chow consumption, plasma glucose and insulin were measured daily. Following 2 days of treatment, mice were anesthetized with 50 mg/kg ketamine hydrochloride/xylazine (Bimeda-MTC Animal Health Inc., Cambridge, ON, Canada/Bayer Inc., Toronto, ON, Canada), a laparotomy was performed and 25 mU/kg human insulin was infused in the portal vein using an ultrafine syringe

and needle (28 G). Two min following the insulin infusion, the epididymal fat pads, soleus, plantaris, and gastrocnemius muscles and liver samples were excised and snap-frozen for analysis.

Immunoblotting of IR and PTPs

For analysis of tyrosine phosphorylation of the β-subunit of IR (IRβ), 2 mg protein lysates from tissues were resuspended in 400 μL lysis buffer and immunoprecipitated by incubation at 4°C overnight with an antibody to IRβ (Santa-Cruz Biotechnology, Inc., Santa Cruz, CA) and protein A conjugated protein A/G (Pierce Chemical Company, Rockford, IL). Immunoprecipitates were washed 3 times with lysis buffer (50 mM Hepes, 1% Triton-X, 2 mM EDTA, 200 mM NaF, 10 mM $Na_2P_4O_7$, 1 mM phenylmethylsulfonylfluoride, 1 mM Na_3VO_4, 10 μg/ml protease inhibitor cocktail) and then boiled in sample buffer (1 M TrisCl, pH 6.8, 30% v/v glycerol, 5% v/v SDS, 0.1% wt/v bromophenol blue, 5% v/v β-mercaptoethanol, all reagents from Sigma-Aldrich Canada Inc., Oakville, ON, Canada). The resulting supernatants were separated on an 8% SDS-PAGE gel and transferred overnight at 4°C to nitrocellulose membrane (Osmonics Inc., Westborough, MA). The membranes were blocked in TBS buffer with Tween-20 (20 mM Tris, 150 mM NaCl, 0.1% Tween-20) containing 5% bovine serum album (all reagents from Sigma-Aldrich Canada Inc.). Immunoblotting was performed using a monoclonal mouse anti-phosphotyrosine antibody (PY99; sc-7020, Santa Cruz Biotechnology, Inc.). Blots were washed and incubated with a goat anti-mouse secondary antibody conjugated to horseradish peroxidase (HRP)(sc-2005, Santa Cruz Biotechnology, Inc.). Protein content was visualized by enhanced chemiluminescence (ECL detection kit, Amersham Pharmacia Biotech, Uppsala, Sweden) and densitometric values were determined with Molecular Dynamics ImageQuant software (Amersham Pharmacia Biotech). IR protein amounts were normalized by re-blotting the membrane with rabbit-polyclonal antibodies to carboxy terminus of IRβ chain of human origin (sc-711, Santa Cruz Biotechnology, Inc.) after

'stripping' the membrane in buffer containing 100 mM β-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl, pH 6.7 at 50°C for 30 min. Blots were washed and incubated with a donkey antirabbit secondary antibody conjugated to HRP (NA934, Amersham Pharmacia Biotech). Protein content was visualized as described above.

For analysis of PTP1B and SH-containing phosphatase 2 (SHP2), protein lysates were run on 10% SDS-PAGE gel and transferred overnight at 4°C to nitrocellulose membrane. Immunoblotting was performed as described above using either a goat polyclonal antibody to PTP1B of human origin (sc-1718, Santa Cruz Biotechnology, Inc.) and rabbit polyclonal antibody to SHP2 of human origin (sc-1119, Santa Cruz Biotechnology, Inc.). Secondary antibodies used were HRP-conjugated, rabbit anti-goat (AP106P, Chemicon International Inc., Temecula, CA) and donkey anti-rabbit, respectively.

Construction and propagation of recombinant adenovirus

The recombinant adenovirus containing PTP1B cDNA, generously provided by Drs. Junfeng Wang, Jay K. Kolls, Michael Bryer-Ash (15, 16), was propagated and titered as previously described (17).

PTP1B overexpression in CHO-ObRb cells

Chinese hamster ovary (CHO) cells stably transfected with the gene for the long form of the leptin receptor, ObRb, were a kind gift from Dr. Takashi Murakami (18). Cells were cultured at 37°C, 5% CO2/95% air in Ham's F-12K nutrient mixture with 2 mM L-glutamine and 1.5 g/L sodium bicarbonate, supplemented with 10% fetal bovine serum (FBS; all reagents from Life Technologies Inc.). For infection studies, cells were grown to approximately 60% confluency in 100 mm plates. Culture media was then replaced with 2 mL of Dulbecco's phosphate buffered saline (D-PBS)(Life Technologies Inc.) with 4.3 mM calcium (D-PBS-Ca^{2+}) containing $6.7x10^9$ plaque forming units (PFU) of adenoviruses expressing either β-galactosidase (Adβ-gal) or PTP1B (AdPTP1B)

for 15 min at room temperature. Fresh culture media was then added to the plates and cells were returned to the CO_2 incubator at 37°C. Following a 48 h infection period, cells were incubated in serum-free media for 4 h and then treated with vehicle or recombinant murine leptin (10 ng/mL) for 15 min. At the end of the treatment period, cells were washed twice in ice-cold D-PBS-Ca^{2+}, then lysed. Analysis of tyrosine phosphorylation of STAT3 was performed as previously described (17). For immunocytochemical detection of STAT3, CHO-ObRb cells were infected with either Adβ-gal or AdPTP1B viruses as described above and then cultured on glass coverslips for 48 h. They were then incubated in serum-free media for 4 h prior to treatment with leptin (10 ng/ml) for 30 min at 37°C and then visualized for STAT3 immunoreactivity as previously described (17).

Adenovirus administration in ob/ob mice

Nine-month old C57BL/6J-*Lepob* mice (Jackson Laboratories) were maintained by the University of Alberta Health Sciences Lab Animal Services and University of British Columbia Animal Services. The animals were housed individually with a 12 h light-dark cycle. The techniques used in this study were in compliance with the guidelines of the Canadian Council on Animal Care and were approved by the Health Sciences Animal Policy and Welfare Committee, University of Alberta, and the University of British Columbia Council on Animal Care. Under brief isofluorane inhalational anaesthesia, mice received 100 µL containing 1 x 10^9 PFU of AdPTP1B or Adβ-gal by tail vein administration. Body weight, food consumption, and plasma glucose were monitored 2 and 5 days after virus injection and blood was collected for the measurement of plasma insulin on day 2 post-virus administration. On day 6 after receiving virus, mice were given either PBS or 0.5 µg/g recombinant murine leptin (PeproTech Inc., Rocky Hill, NJ) by IP injection, twice daily for 2 days (at 900 h and 1600 h). The dosage of leptin used in *ob/ob* mice was lower than in lean mice due to the hypersensitivity of *ob/ob* mice to leptin. We have previously observed that this dose of leptin does not affect body weight after 2 days of administration but does

affect food consumption (17). Body weight, food consumption, plasma glucose and insulin were measured daily. Six hours following the last treatment, mice were anesthetized by inhalation of isofluorane and a laparotomy was performed. Human insulin (25 mU/kg) was infused in the portal vein using a syringe and ultrafine needle (28 G). Two min following the insulin infusion, the epididymal fat pads, soleus, plantaris, and gastrocnemius muscles and liver samples were excised and snap-frozen in liquid N_2 for further analysis. Small blocks of tissue were also removed from Adβ-gal infected mice and immediately embedded in Tissue-Tek OCT compound (IMEB Inc., San Marcos, CA) and immersed slowly in CO_2/ethanol above liquid N_2 for cryosection and staining for β-galactosidase expression.

β-galactosidase detection in tissues from adenovirus infected ob/ob mice

Tissues were processed into 10 μm sections and set onto glass microscope slides. Tissues were washed in PBS, fixed with 0.2% glutaraldehyde for 5 min, then washed in PBS again before incubatation for 4 h at 37°C with X-gal solution (1 mg/mL 5-Bromo-4-chloro-3-indolyl b-D-galactopyranoside (Promega Corp., Madison, WI), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide, 2 mM $MgCl_2$, all in PBS) (all reagents from Sigma-Aldrich Canada Inc.). Following the incubation period, slides were washed twice in PBS and tissues were examined with a Leica DMIRB microscope (Leica Microsystems, Germany). Images were captured via a CoolSnap camera (RS Photometrics, Tucson, AZ) and areas of β-galactosidase staining were determined using Iplab software (Scanalytics, Inc., Fairfax, VA).

Statistical analysis

Group differences were evaluated by paired t-test or ANOVA analyses and Tukey post-hoc tests with GraphPad Prism software (GraphPad Software Inc., San Diego, CA), with $P<0.05$ deemed significant.

4.3. Results

High fat feeding induces hyperglycemia, hyperinsulinemia and hyperleptinemia in C57BL/6 mice

Daily caloric intake by mice on the HF diet was slightly elevated compared to mice on the LF diet, resulting in ~ 15% increase in cumulative calories consumed after 72 days on the diets (912.5± 31.1 vs 788.5 ± 36.5 kcal)(Fig. 4.1). At the end of the 72 day period, HF fed mice weighed ~ 25% more than LF fed mice (35.5 ± 0.6 vs 28.0 ± 1.2 g). Differences in body weight became significant by day 18 on the diets (HF; 25.1 ± 0.3 vs LF; 23.5 ± 0.7 g, p=0.024) while changes in cumulative food intake were noticeable by day 16 (HF; 201 ± 1.7 vs LF; 186.3 ± 4.0 kcal, p=0.0017). Since weight gain can be associated with insulin resistance and elevated leptin concentrations, plasma glucose, insulin, and leptin levels were determined from samples taken 35 days after initiation of diets. Consistent with the development of insulin resistance, plasma glucose and insulin were both elevated in HF fed mice by 23% and 59%, respectively, and leptin values in HF fed mice were elevated 6 times those of LF fed mice, a reflection of increased body fat (Table 4.1).

High fat feeding reduces the effects of leptin on food consumption in C57BL/6 mice

Prior to leptin treatment 10-weeks into the HF diet, HF fed mice had body weights that were ~25% higher than the body weights of LF fed mice (Table 4.2). Mice were allocated to receiving PBS or leptin (2.5 μg/g) twice daily for 2 days. After 2 days of treatment, there were no changes in body weights in any treatment group. However, leptin reduced food consumption by 50% following 2 days of treatment in LF fed mice. In comparison, PBS treated LF fed mice displayed no changes in food consumption. Although leptin suppressed feeding in LF fed mice, it had no effect on food intake in HF fed mice (Table 4.2). At this dose of leptin, serum glucose concentrations were not affected in either group during 2 days of treatment. However, leptin dramatically reduced plasma insulin levels

after day 2 by ~50% in LF fed mice and ~40% in HF fed mice. While consistent with improvements of insulin sensitivity, the improvement was not to the same level in the HF fed mice as insulin levels remained more than 3 times higher in this group compared to the LF fed leptin-treated group.

High-fat feeding leads to hepatic insulin resistance and elevated PTP1B protein expression

Consistent with an insulin resistant state, mice on HF diet had reduced IR phosphorylation compared to mice on LF diet (pTyr/IR densitometric ratios for HF vs LF; 0.82 ± 0.06 vs 0.54 ± 0.16, $P<0.05$)(Fig. 4.2). Since phosphatases have been implicated as negative regulators of both insulin and leptin signalling, we determined whether high fat feeding affects the expression levels of PTP1B and another phosphatase, Src homology 2-containing phosphatase (SHP2) in liver, skeletal muscle and adipose tissue. While there were no significant differences in PTP1B expression in adipose tissue between HF and LF fed mice, expression of PTP1B in muscle was doubled in the HF fed mice and liver PTP1B expression was 6.4 fold that of the LF group (Fig. 4.3). The difference in PTP1B expression appeared to be phosphatase specific, since there were no differences in the expression of muscle or liver SHP2 in HF compared to LF fed mice.

PTP1B overexpression in CHO-ObRb cells inhibits STAT3 phosphorylation and nuclear translocation by leptin

While leptin treatment resulted in ~10 fold higher STAT3 phosphorylation in comparison to vehicle treatment in control Adβ-gal infected cells, PTP1B overexpression suppressed this effect (Fig. 4.4A). Furthermore, in contrast to Adβ-gal infected cells, PTP1B overexpression excluded STAT3 from the nucleus, even in the presence of leptin (Fig. 4.4B).

Adenovirus-mediated overexpression of PTP1B in livers of ob/ob mice has minimal effects on glucose homeostasis for 5 days post-infection

To directly assess the *in vivo* impact of elevated hepatic PTP1B expression on leptin sensitivity, glucose homeostasis, body weight and food intake, adenovirus expressing the PTP1B gene (AdPTP1B) or β-galactosidase as control (Adβ-gal) was administered to leptin sensitive but deficient *ob/ob* mice, by tail-vein injection. Following administration of AdPTP1B or Adβ-gal, there was a decrease in food intake intake, most likely attributed to effects of the procedure (Table 3). However, food consumption continually increased in the 5 day post-infection period and by day 5, only a small decrease in food intake was observed. There were also no differences in body weight, plasma glucose or insulin levels compared to pre-virus values (Table 3). In order to determine the tissue distribution of virus, tissue was extracted from β-gal infected mice and β-gal staining was performed. Approximately 40% of hepatocytes showed intense blue staining while there was minimal staining in all other tissues examined (Fig. 4.5). In particular, β-gal staining was not detected in pancreatic samples or sections containing hypothalamic nuclei. Liver tissue was also extracted for determination of PTP1B protein levels. In AdPTP1B mice, liver PTP1B protein levels were approximately 5 times greater than in mice receiving Adβ-gal virus, confirming liver overexpression of PTP1B (Fig. 4.6A).

Increased PTP1B expression in livers of ob/ob mice inhibits the effects of leptin on food consumption and blood glucose concentrations

Five days post-infection, mice were subjected to PBS or leptin (0.5 μg/g) treatment by IP injection, twice daily for 2 days. Leptin, at this dose and administration period, had no effect on body weight in either group but significantly reduced food intake by ~50% in animals that received Adβ-gal (Table 3). In comparison, food consumption in AdPTP1B infected mice was only reduced by 20%, not reaching statistical significance. Therefore, overexpression of PTP1B in the liver alone suppressed the ability of leptin to induce satiety in

ob/ob mice. Similarly, leptin reduced fasting serum glucose concentrations by ~50% in Adβ-gal mice ($P<0.05$) and ~20% in AdPTP1B mice (not significant), indicating a role of PTP1B in preventing leptin from normalizing high glucose levels. Interestingly, leptin was equally capable of reducing serum insulin concentrations in both Adβ-gal and AdPTP1B mice to ~35% of day 0 concentrations. Additionally, we looked at whether PTP1B overexpression affected leptin mediated improvements in insulin sensitivity at the level of IR in liver. IR phosphorylation in response to insulin, an early step in the insulin signalling cascade is dramatically reduced in insulin resistant *ob/ob* mice compared to wild-type lean, insulin sensitive mice, and is virtually normalized following 2 days leptin treatment (17). Following the final leptin or PBS treatment on day 2, mice were fasted for 6 h, then given an infusion of insulin (25 mU/kg) via the hepatic portal vein. In leptin-treated Adβ-gal mice, insulin induced IR phosphorylation. In contrast, insulin was unable to stimulate IR phosphorylation in leptin-treated AdPTP1B (Fig. 4.6B).

4.4. Discussion

Although type 2 diabetes and obesity are becoming epidemic like in prevalence, the polygenic character and multiple insult nature of the diseases hamper progress in the development of effective treatments. There is a strong association of type 2 diabetes with obesity such that approximately 80% of patients with type 2 diabetes are obese (19). Obesity sets the stage for the onset of insulin resistance and impaired glucose tolerance on a genetically susceptible background. Concurrent failure of pancreatic β cells to produce sufficient insulin precipitates further deterioration of glucose control and eventually diabetes (20-22). Both insulin and leptin resistance are hallmarks of obesity and risk factors for the development of diabetes, yet a common mechanism linking the resistance to these two hormones is yet to be identified. In agreement with earlier studies (11, 12), we find that in addition to the well-characterized function of PTP1B as a negative regulator of insulin signalling, PTP1B is also a negative regulator of leptin signalling pathways. The dual role of PTP1B as both an inhibitor of insulin and leptin signalling implicates PTP1B as a common mediator of the resistance to both hormones and thus a potential link between obesity and diabetes. While the full spectrum of PTP1B substrates and actions is not yet known, there is a great deal of interest in targeting this phosphatase as a possible therapy for obesity and type 2 diabetes (13, 14).

In this study we sought to determine the extent to which PTP1B might contribute to the onset of insulin and leptin resistance. The recent surge in childhood obesity (23) and the increasing prevalence of type 2 diabetes in children (24) underscores findings that excessive caloric intake and insufficient physical activity contribute to this disease state (25). We modeled the development of human obesity in mice by feeding a high fat (HF) diet. Body weight and daily food consumption were monitored for 10 weeks and changes in plasma glucose, insulin, and leptin levels were assessed 5 weeks after initiation of diets. As expected, relative to mice maintained on a low fat (LF) diet, HF fed mice gained more weight, consumed more calories and were hyperglycemic,

hyperinsulinemic and hyperleptinemic by 5 weeks of the study. Increased body weight gain of HF fed mice relative to LF fed mice started at approximately 18 days into the diets while measurable changes in food intake were detected by day 16, likely indicating the development of resistance to the satiety actions of leptin. The mechanism by which leptin resistance develops following regular consumption of a high fat diet is yet unknown. Some factors that may contribute include leptin receptor down-regulation, reduced leptin transport into the brain, or activation of Suppressors of Cytokine Signalling (SOCS) which inhibit leptin signalling through JAK-STAT (1, 2, 26).

We explored the hypothesis that up-regulation of the phosphatase, PTP1B may be involved in the development of leptin resistance. After 10 weeks on the diets, at which point HF fed mice displayed insulin resistance and resistance to leptin mediated reductions in caloric consumption, tissues were extracted and assessed for PTP1B protein expression. PTP1B expression was not elevated in adipose tissue of HF fed mice, but was elevated in muscle, and most dramatically in liver relative to the LF fed groups. Consistent with the notion that PTP1B is a negative regulator of insulin signalling, we found that the elevated hepatic expression of PTP1B was associated with a reduced ability of insulin to activate IR phosphorylation in hepatocytes. To assess what contribution elevated hepatic PTP1B might have in the development of leptin resistance, we overexpressed PTP1B in livers of leptin deficient *ob/ob* mice. These animals are typically severely insulin resistant despite lower than normal hepatic levels of PTP1B (17) and display enhanced sensitivity to both the weight reducing and glucose lowering actions of leptin (27-30). Nine month old *ob/ob* mice weighed approximately twice that of wild-type mice and displayed markedly elevated fasting plasma glucose and insulin concentrations. Overexpression of PTP1B in liver did not alter body weight, food consumption or plasma concentrations of glucose and insulin during the 1 week study period. Furthermore, it did not overly curtail leptin-mediated reductions of plasma insulin, a process that involves central actions and direct effects of leptin on pancreatic β cells (31) in

addition to improvements in insulin sensitivity (17). However, elevated hepatic PTP1B expression markedly impaired the ability of leptin to reduce plasma glucose levels. The mechanism by which leptin reduces blood glucose levels likely involves enhanced glucose uptake by peripheral tissues and inhibition of hepatic glucose production (32-36). While we have previously demonstrated that leptin can improve insulin sensitivity by direct actions on hepatocytes (17), we cannot exclude the possibility that the improvement in hepatic insulin sensitivity following leptin injection observed in this study is also centrally mediated. Indeed it has recently been demonstrated that intracerebroventricular administration of leptin can improve hepatic insulin sensitivity, presumably via CNS pathways (37). However, it is noteworthy that gold thioglucose destruction of hypothalamic leptin receptor expressing neurons in PTP1B$^{-/-}$ mice has no impact on the enhanced insulin sensitivity of these animals (12). Therefore, leptin signalling in the hypothalamus does not appear to be required for regulation of insulin sensitivity by PTP1B. Our findings confirm that liver is an important target organ for the glucose lowering action of leptin and provides novel evidence that this pathway is negatively regulated by hepatic PTP1B.

Interestingly, increased hepatic PTP1B expression in *ob/ob* mice was also able to markedly inhibit leptin-induced satiety. In control *ob/ob* mice receiving Adβ-gal, exogenous leptin reduced food consumption by approximately 50% after 2 days of treatment. However, in AdPTP1B treated mice, PTP1B overexpression in hepatocytes reduced the effectiveness of leptin in suppressing food consumption by more than half. While traditionally the regulatory effect of leptin on food intake is viewed as a response to direct central actions of leptin (1, 2, 38, 39), our results provide the first evidence that leptin action in the liver can regulate feeding behavior. Furthermore, our findings are supportive of the notion of Zabolotny *et al.* that a large portion of the influence of PTP1B on body mass regulation must be outside of leptin responsive hypothalamic neurons (12). If this occurs as a result of direct actions of leptin on hepatocytes, as opposed to indirectly via signals originating in hypothalamic leptin-sensitive neurons,

analysis of mice with chronic liver-specific reduction in leptin receptor expression suggests that with life-long deficiency, normal control of food intake can be maintained by other redundant pathways (39). Although the liver has long been implicated in the control of food intake and body weight regulation, the pathways and signalling molecules involved have not been definitively established (reviewed in (40)). One theory is that an increase in glucose utilization can produce a satiating effect (41). Sensors in the liver that detect changes in fuel metabolism may trigger a signal in vagal afferents that act centrally to inhibit food intake (42). Leptin has a lipolytic effect in the liver, depleting triglyceride content, and increasing free fatty acid oxidation (43-45). Increased hepatic fatty acid oxidation by leptin may provide an alternate satiety signal that could also be relayed through vagal afferents (46). Whether leptin signalling through a PTP1B sensitive pathway suppresses food intake via a vagally mediated pathway remains to be determined.

In summary, our findings demonstrate that high fat feeding leading to insulin and leptin resistance is associated with an increase in PTP1B protein expression in liver. We also find that overexpression of PTP1B in liver curtails the ability of leptin to lower blood glucose levels and suppress food intake. Therefore, we propose that leptin signalling in liver is important not only in regulating glucose homeostasis, but also food intake and energy balance. Our discovery that PTP1B inhibits these actions of leptin indicates that hepatic PTP1B could be an important component of the development of both insulin and leptin resistance, and thus a link in the pathogenesis of obesity and diabetes. Previously we determined that exposure of hepatocytes to leptin can increase PTP1B expression (17). Taken together, it is tempting to speculate that a diet high in fat can promote diabetes associated with obesity by elevating fat stores and thus circulating leptin levels, which initially protect nonadipocytes from steatosis and lipotoxicity (3), but eventually precipitate both insulin and leptin resistance through increased expression of PTP1B. While such a pathway appears counter-productive from a physiological perspective, it might be considered maladaptive

given the concept that leptin evolved to signal declining fat stores, and less so excessive fat deposition (47). Thus such proposed communication between fat and liver involving leptin and PTP1B might be designed as a mechanism by which negative energy balance facilitates an advantageous improvement of insulin sensitivity. This hypothesis warrants further investigation. Finally, our observations suggest that strategies aimed at suppressing PTP1B specifically in liver could be envisaged to improve both hepatic insulin and leptin sensitivity and thereby improve both glucose homeostasis and regulation of food intake. Further understanding of the role of PTP1B in the development of leptin and insulin resistance may provide additional insight into the pathophysiology of obesity and diabetes and reveal novel therapeutic strategies to treat these debilitating diseases.

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Table 4.1. High fat feeding induces hyperglycemia, hyperinsulinemia and hyperleptinemia in C57BL/6 mice

Parameter	Diet (% coconut oil)	
	11%	58%
Glucose (mM)	11.4 ± 0.3	14.0 ± 0.4*
Insulin (pM)	145.6 ± 20.7	232.0 ± 14.3*
Leptin (ng/mL)	1.1 ± 0.2	6.3 ± 0.8*

C57BL/6 mice were fed ad libitum diets consisting of either 11% or 58% coconut oil comprising total calories. After 35 days on the respective diets, mice were fasted for 4 h and blood was collected for measurement of fasting plasma glucose, insulin and leptin concentrations. Data are means ±SE. *$P \leq 0.05$. 11%: n=8, 58%: n=11.

Table 4.2. High fat diet reduces the effects of leptin on food consumption in C57BL/6 mice

			Leptin treatment period		
Parameter	**Diet**	**Treatment**	**Day 0**	**Day 1**	**Day 2**
Body weight (g)	11%	PBS	27.1 ±1.4	27.7 ± 2.1	27.4 ± 1.9
	11%	Leptin	28.8 ± 2.2	28.6 ± 1.9	27.3 ± 1.8
	58%	PBS	33.6 ± 0.6	33.7 ± 0.6	33.1 ± 0.6
	58%	Leptin	36.5 ± 0.4	36.8 ± 0.4	36.5 ± 0.3
Food intake (kcal/d)	11%	PBS	11.8 ± 0.8	12.2 ± 0.0	11.4 ± 0.0
	11%	Leptin	10.6 ± 0.4	10.2 ± 00	5.3 ± 0.4*†
	58%	PBS	12.2 ± 0.6	11.1 ± 0.6	10.0 ± 0.6
	58%	Leptin	13.9 ± 1.1	12.8 ± 0.6	11.1 ± 0.6
Plasma glucose (mM)	11%	PBS	9.5 ± 0.9	6.9 ± 0.7	7.8 ± 0.8
	11%	Leptin	10.1 ± 0.6	10.8 ± 1.1	9.9 ± 1.0
	58%	PBS	10.9 ± 0.4	11.0 ± 0.6	11.6 ± 1.8
	58%	Leptin	11.9 ± 0.6	10.6 ± 1.0	9.2 ±1.0
Plasma insulin (pM)	11%	PBS	133 ± 8	145 ± 29	204 ± 38
	11%	Leptin	125 ± 22	110 ± 8	60 ± 3*
	58%	PBS	259 ± 64	192 ± 38	330 ± 46
	58%	Leptin	370 ± 57	427 ± 37	220 ± 23*

C57BL/6 mice were fed ad libitum diets consisting of either 11% or 58% coconut oil comprising total calories for 72 days at which point PBS or leptin (2.5 μg/g, twice daily) was administered for 2 days. Day 0 indicates values prior to initial leptin injection. All parameters were measured daily after mice were fasted for 4 h. Data are means ±SE. *$P \leq 0.05$ vs Day 0 of same treatment group, †$P \leq 0.05$ vs Day 1 of same treatment group. 11% PBS: n=4, 11% leptin: n=4, 58% PBS: n=5, 58% leptin, n=6.

Table 4.3. Increased PTP1B expression in livers of *ob/ob* mice inhibits the effects of leptin on food consumption and blood glucose concentrations

		Adenovirus administration			Leptin treatment period		
	Treatment	Day 0	Day 2	Day 5	Day 0	Day 1	Day 2
BW (g)	AdPTP/PBS	51.3 ± 3.2	51.7 ± 3.2	51.9 ± 3.0	51.4 ± 3.1	52.0 ± 3.0	51.9 ± 3.0
	AdPTP/leptin	59.8 ± 4.3	60.4 ± 4.2	60.3 ± 3.9	59.8 ± 4.1	59.9 ± 4.0	58.9 ± 3.8
	Adβ-gal/leptin	69.7 ± 5.3	69.6 ± 5.4	69.4 ± 5.3	69.5 ± 5.3	69.0 ± 5.5	68.1 ± 5.5
Food intake	AdPTP/PBS	5.6 ± 0.2	3.5 ± 0.3*	4.3 ± 0.5	4.1 ± 0.5	4.6 ± 0.4	4.2 ± 0.3
(kcal/d)	AdPTP/leptin	5.3 ± 0.4	4.0 ± 0.4	4.4 ± 0.3	4.4 ± 0.2	4.0 ± 0.5	3.5 ± 0.4
	Adβ-gal/leptin	5.4 ± 0.3	3.9 ± 0.6	5.1 ± 0.6	5.0 ± 0.6	3.9 ± 0.7	2.8 ± 0.5*
Glucose	AdPTP/PBS	18.9 ± 3.6	23.7 ± 4.2	24.1 ± 4.1	23.9 ± 4.0	25.9 ± 5.0	21.9 ± 4.0
(mM)	AdPTP/leptin	20.8 ± 4.1	23.1 ± 3.1	20.2 ± 2.6	21.7 ± 3.6	16.8 ± 2.3	16.9 ± 1.8
	Adβ-gal/leptin	22.9 ± 3.4	25.5 ± 2.9	23.2 ± 3.4	24.1 ± 2.9	19.3 ± 3.0	13.9 ± 2.0*
Insulin	AdPTP/PBS	9.9 ± 4.5	6.9 ± 2.3	Not	7.5 ± 2.7	10.4 ± 3.7	7.3 ± 2.5
(nM)	AdPTP/leptin	9.7 ± 3.2	11.9 ± 3.2	measured	10.5 ± 3.2	6.7 ± 2.1	3.6 ± 1.3*
	Adβ-gal/leptin	12.5 ± 5.1	12.1 ± 1.4		13.7 ± 3.2	9.3 ± 3.2	4.9 ± 1.6*

Ob/ob mice (8 months old) received $1x10^9$ PFU of Adβ-gal or AdPTP1B on Day 0. Body weight, food intake and plasma glucose and insulin levels were measured prior to virus injection (Day 0), and 2 and/or 5 days post-adenoviral injection. On the sixth day after adenovirus injection, PBS or leptin (0.5 μg/g, twice daily) was administered for 2 days. Day 0 of leptin treatment period indicates values prior to initial leptin injection. All parameters were measured daily after mice were fasted for 4 h. Data are means ±SE. *$P \leq 0.05$ vs Day 0 of same treatment group. AdPTP1B/PBS: n=4, AdPTP1B/leptin: n=6, Adβ-gal/leptin: n=7.

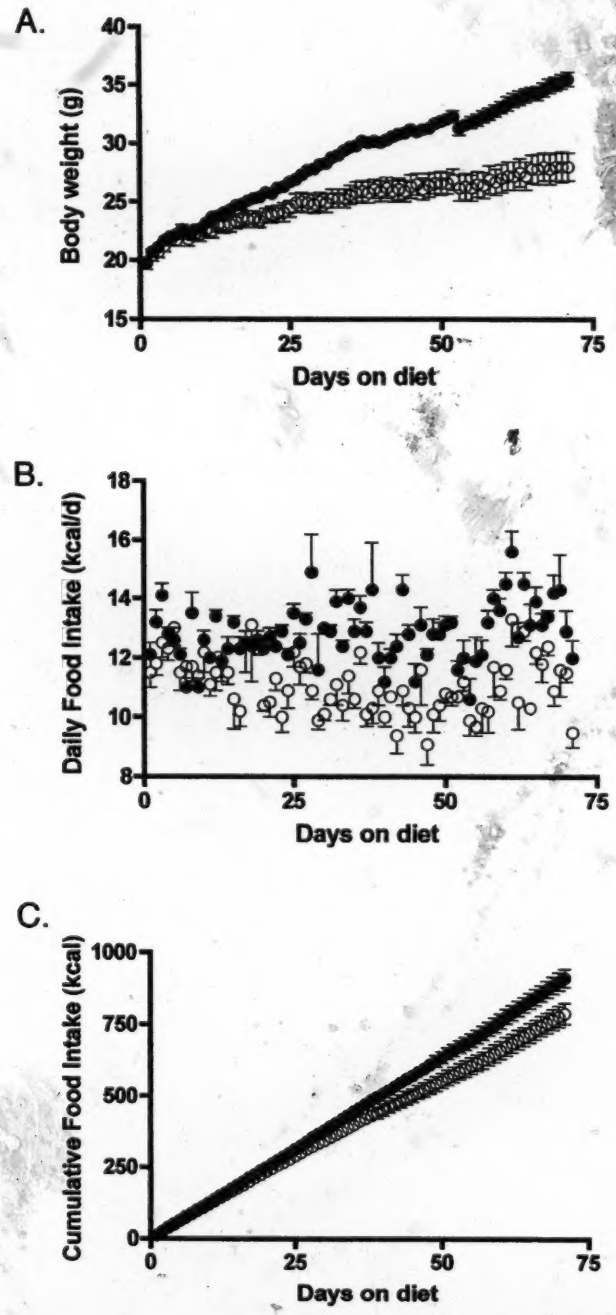

Figure 4.1. High fat feeding increased body weight and food consumption. C57BL/6 mice were fed ad libitum diets consisting of either low fat (LF) or high fat (HF). Body weight and food consumption was measure daily for 72 days. A. Body weight gain and B. Caloric consumption in mice fed diets consisting of LF (open circles, n=8) and HF (closed circles, n=11) plotted as mean ± SEM. * denotes differences observed between mice on LF and HF diets on the same day. *P<0.05

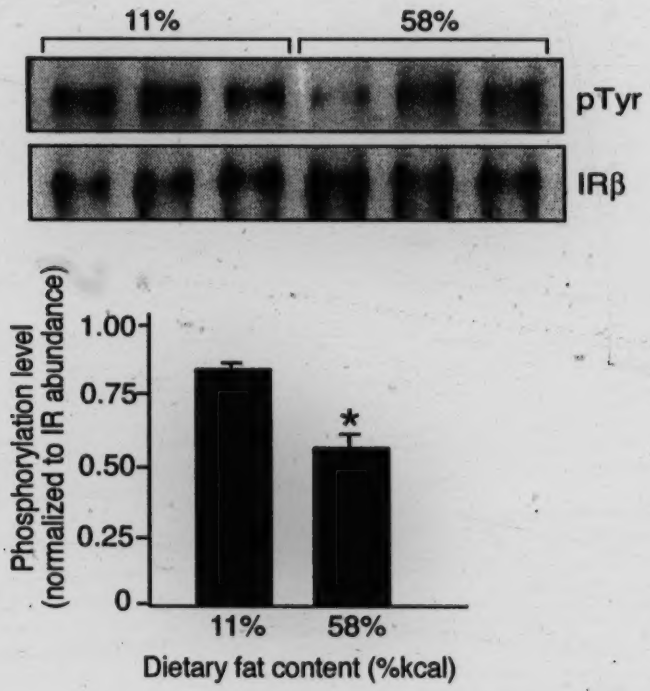

Figure 4.2. High fat feeding decreases insulin-stimulated IR phosphorylation in liver. C57BL/6 mice fed either low fat (11%) or high fat (58%) diets were fasted for 6h, then given an infusion of insulin (25 mU/kg) via the hepatic portal vein. Two min following the insulin infusion, liver tissue was harvested and protein lysates prepared were immunoprecipitated with antibody to IR. Phosphorylation of IR was assessed by immunoblotting with anti-phosphotyrosine antibody. Membranes were stripped and reprobed with IR antibody. Representative immunoblots depicting tyrosine phosphorylation of a 95 kDa IR β-subunit is shown. Below each immunoblot is displayed the intensities (arbitrary units) of phosphotyrosine expression, corrected for total IR (n=3, *$P<0.05$)

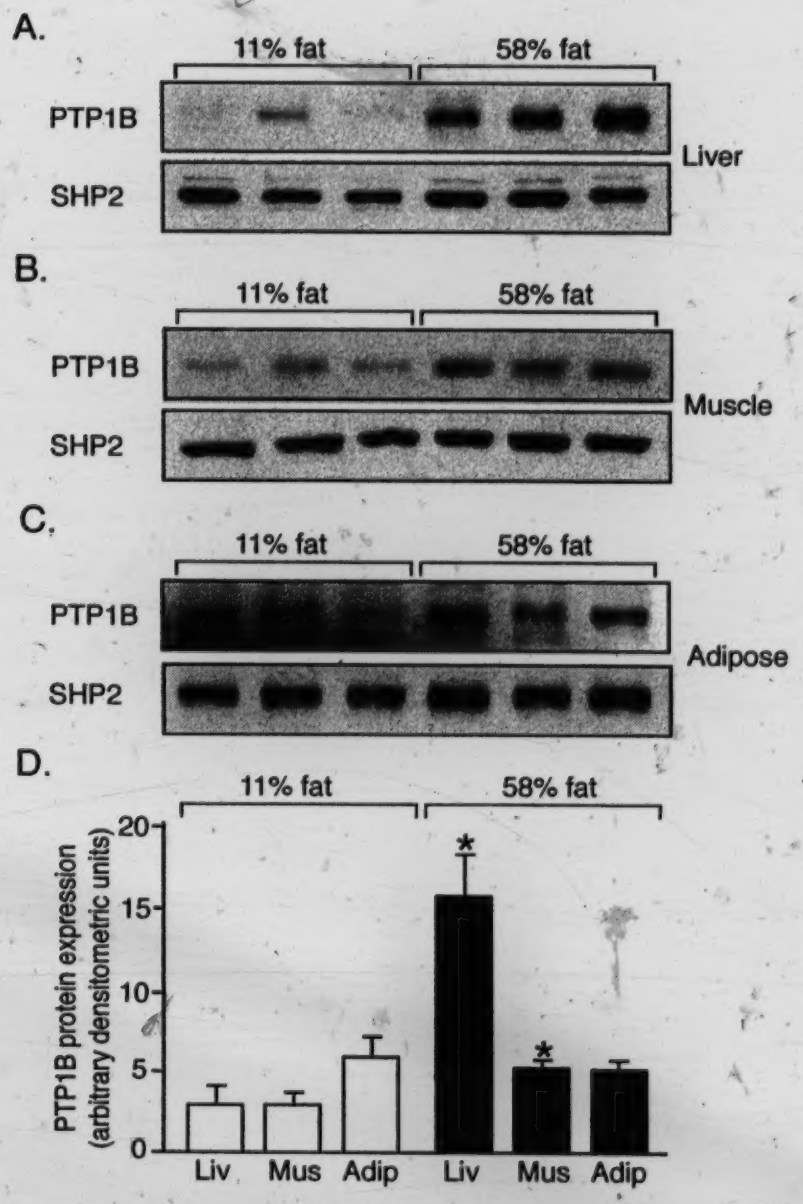

Figure 4.3. High fat feeding increases PTP1B expression in a tissue and phosphatase specific manner. Protein lysates prepared from tissues extracted from C57BL/6 mice after 10 weeks of feeding on either low fat (LF) or high fat (HF) diets were immunoblotted with antibodies to either PTP1B or SHP2. Immunoblots showing PTP1B (50 kDa) and SHP2 (68 kDa) protein expression are shown for A. liver, B. skeletal muscle, and C. adipose tissue. D. Densitometric analysis of PTP1B protein expression in immunoblots A, B, and C were quantitatively determined. Densitometric averages in arbitrary units are displayed for liver, muscle and adipose tissue from mice on LF and HF diets. (n=3. *P<0.05)

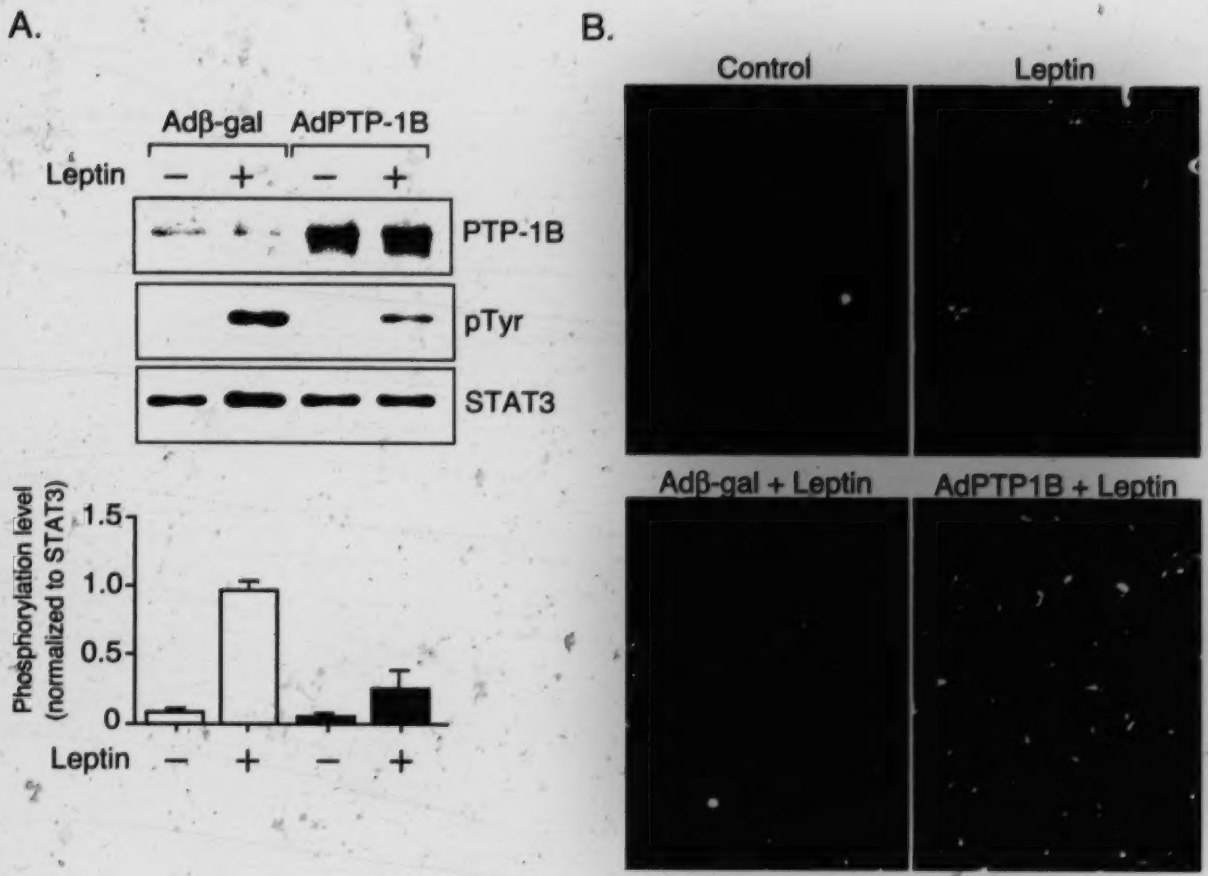

Figure 4.4. PTP1B over-expression in CHO-ObRb cells inhibits STAT3 phosphorylation and nuclear translocation by leptin. CHO-ObRb cells were infected with Adβ-gal or AdPTP1B for a 48 h period. Cells were then incubated in serum-free media for 4 h and treated with vehicle or recombinant murine leptin (10 ng/ml) for 15 min. A. Protein lysates prepared from infected CHO-ObRb cells were either immunoblotted with anti-PTP1B antibody or immunoprecipitated with antibody to STAT3. Phosphorylation of STAT3 was assessed by immunoblotting with anti-phosphotyrosine antibody. Membranes were stripped and reprobed with STAT3 antibody. A representative immunoblot depicting tyrosine phosphorylation of STAT3 is shown. Below the immunoblots is displayed the mean intensities (arbitrary untis) of phosphotyrosine expression, corrected for total STAT3 protein ± SE (n=). B. Immunofluorescent detection of STAT3 in CHO-ObRb cells either treated with PBS (control), leptin (10 ng/mL) or leptin following infection with Adβ-gal or AdPTP1B.

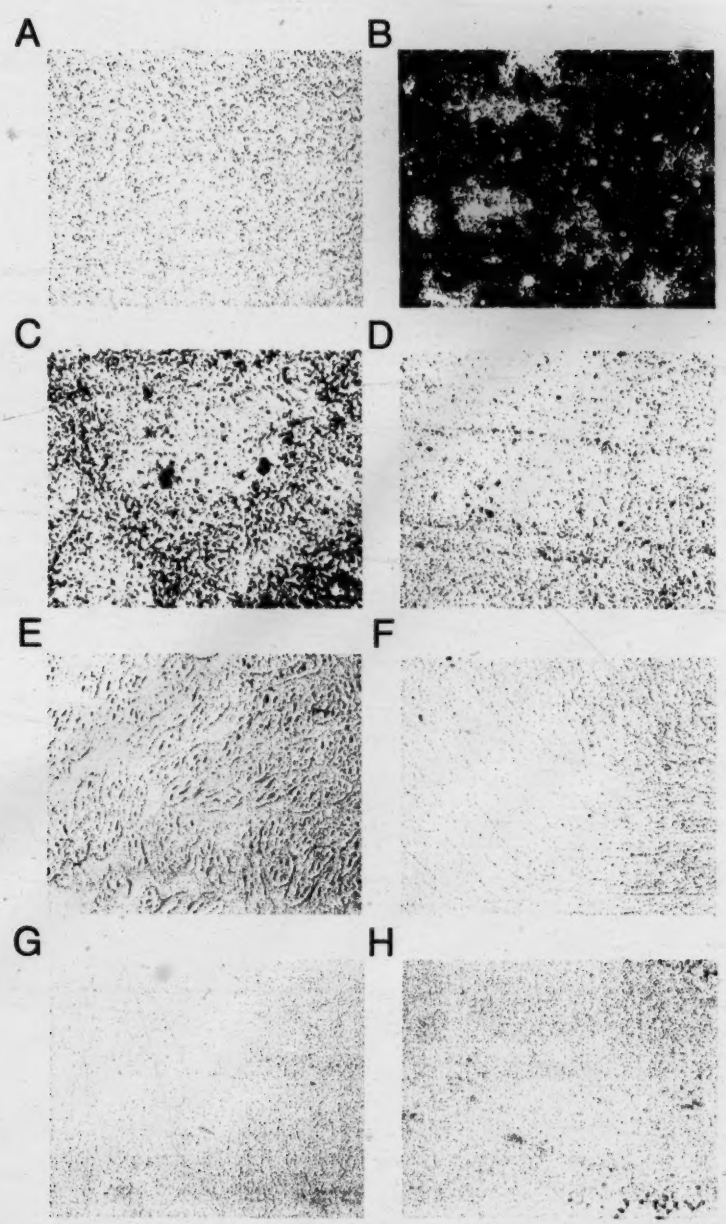

Figure 4.5. β-galactosidase histochemical analysis of mouse tissues. *Ob/ob* mice received a single injection of either saline or $1x10^9$ PFU of Adβ-gal via tail vein. Seven days post-administration, liver tissue was immediately imbedded in Tissue-Tek OCT compound and stained for β-gal expression. Panel A shows a representative image of liver tissue from uninfected mice. Panels B-H are representative images of tissues taken from Adβ-gal infected mice. B. liver, C. pancreas, D. brain, E. muscle, F. heart, G. kidney, H. spleen. Magnification is 100x for all images.

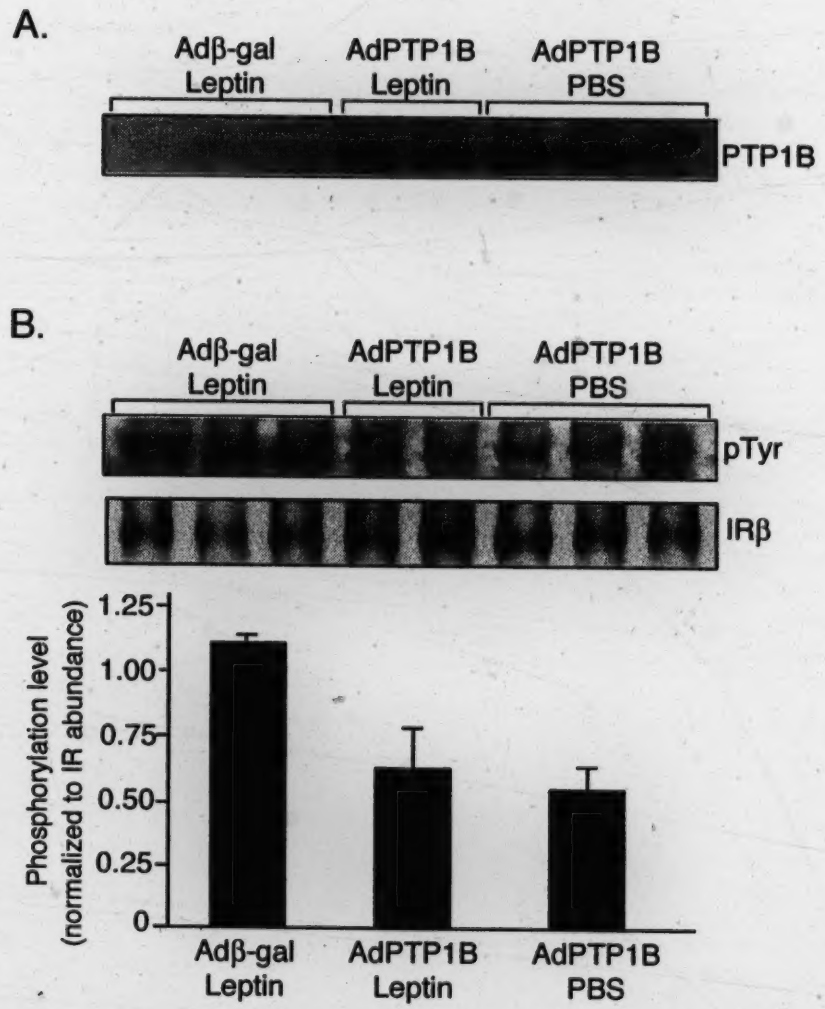

Figure 4.6. PTP1B over-expression in liver decreases insulin stimulated IR phosphorylation in leptin-treated mice. *Ob/ob* mice infected with Adβ-gal or AdPTP1B were treated with leptin or PBS for 2 days. Mice were fasted for 6h, then given an injection of insulin (25 mU/kg) via the hepatic portal vein. Two min following the insulin infusion, tissues were harvested and protein lysates prepared from mice tissue extracts were either A. immunoblotted with anti-PTP1B antibody or B. immunoprecipitated with antibody to IR. Phosphorylation of IR was assessed by immunoblotting with anti-phosphotyrosine antibody. Membranes were stripped and reprobed with IR antibody. Representative immunoblots depicting tyrosine phosphorylation of a 95 kDa IR β-subunit is shown. Below the immunoblots is displayed the mean intensities (arbitrary units) of phosphotyrosine expression, corrected for total IR protein ± SE (n=2).

CHAPTER 5
DISCUSSION

5.1. Obesity and Diabetes Revisited

Type 2 diabetes, once considered a disease of affluent nations, is now positioned as one of the main threats to global human health (1). The main driving force behind the increased incidence is a staggering increase in obesity, the single most important risk factor for the development of diabetes. Pronounced changes in environment, human behavior and lifestyles that have accompanied globalization have resulted in the astounding rise in both obesity and diabetes. Modest amounts of weight loss can significantly delay the progression to diabetes in overweight individuals with impaired glucose tolerance (2-4). As diabetes is closely associated with increasing adiposity, a shared commonality between these two diseases may be a factor secreted from fat cells. Free fatty acids that are produced by hydrolysis of triglycerides and increased production of tumour necrosis factor $\propto$ in adipose tissue can both inhibit glucose utilization in peripheral tissues (5, 6). A host of other fat-derived adipocytokines have since been discovered that could also link obesity with insulin resistance (7, 8). In these studies we have investigated the possibility that leptin, secreted from adipocytes, can regulate glucose homeostasis and that failure in leptin action can exacerbate obesity and propel a predisposed individual into a state of diabetes.

Our understanding of the hormone leptin is continually being revised with the barrage of new information disseminated monthly on this topic. At the time of its discovery, leptin was believed to be an anti-obesity hormone (9-12). Since that time, the theory has evolved and now focuses on leptin as a starvation signal since its levels decline rapidly upon fasting, disproportionately to changes in adipose mass (13-17). Regardless of the evolutionary premise behind its existence, leptin or rather, resistance to leptin, plagues an astounding proportion of the human population (18, 19). The profound effect of leptin on weight loss and feeding behavior is of considerable interest as a marketable diet regiment. Given the critical role of obesity in the development of insulin resistance and

impairment of insulin secretion, successful approaches to diminishing food intake and enhancing energy expenditure will prove of great benefit in targeting the prevention and treatment of diabetes. Independently from its effects on body weight, leptin also regulates glucose and lipid metabolism. The recognition of these multifaceted benefits of leptin confers an advantage for leptin as a therapy for both obesity and diabetes.

As pharmacological approaches for diabetes continues to develop and improve, the goal for treatment is to intervene when early clinical signs first manifest. An ideal drug could target diabetes through the following approaches: (1) induce weight loss, (2) improve β cell function in regulated insulin secretion, (3) reduce excessive production of glucose by the liver, and (4) augment insulin signalling in insulin-responsive tissues. According to our findings, leptin mediates all of these effects and thus presents as a candidate for the treatment of obesity and associated diabetes.

5.2. Summary: Leptin Effects in Pancreatic β cells

It is well established that diabetes is a polygenic disorder in which both hereditary and environmental influences determine onset and severity. Of considerable debate is whether insulin resistance or impaired β cell function is the primary defect. Hyperinsulinemia is classically believed to arise in compensation to an impaired response to insulin in peripheral tissues (20-30). Deterioration in β cell function follows, incurred upon by chronic and increased demands for insulin, resulting in the disease state of diabetes. Due to a number of studies where it was observed that reduced insulin sensitivity was often preceded by a period of hyperinsulinemia (31-34), this traditional belief is now being challenged. Insulin is an anabolic hormone that promotes fatty acid biosynthesis and storage of triglyceride. Hyperinsulinemia may induce insulin resistance to prevent lipid accumulation in non-adipose tissue. Insulin resistance and the impaired ability of muscle and liver to utilize glucose may cue the β cells to

produce even more insulin, further exacerbating the hyperinsulinemia and resulting in the eventual progression to diabetes.

In agreement with results documented by Pelleymounter *et al.* (9) we observed that the metabolic parameters of blood glucose and insulin concentrations were altered by leptin before noticeable changes in body weight were detected in *ob/ob* mice. Leptin reduced plasma glucose and insulin levels even prior to changes in appetite. Hypersecretion of insulin from the pancreas is among the earliest detectable metabolic alterations in some genetically obese animals including the *ob/ob* mouse (35-43). Since hyperinsulinemia may be one of the earliest signs of metabolic instability and impaired β cell function, we investigated whether direct effects on pancreatic β cells may appropriately explain the insulin-lowering effects of leptin. As with diet-induced obesity, an increase in body fat resulting in elevated circulating levels of leptin would relay the message that insulin signalling is adequate and that further release of insulin is no longer required. This pathway would serve to regulate fat deposition since insulin is a major adipogenic hormone. In earlier studies, it was determined that one mechanism by which leptin suppressed insulin secretion was by opening of K_{ATP} channels and hyperpolarizing β cells (44-46). We determined that the opening of K_{ATP} channels by leptin may be associated with a decrease in $[ATP]_i$ in INS-1 β cells. A reduction in glucose availability for metabolism may explain the decreased $[ATP]_i$. The reduction of intracellular ATP in response to glucose suggested that leptin interfered with substrate-driven induction of insulin secretion. Reductions in basal insulin secretion was not mediated by this pathway although another mechanism, such as protein kinase C, may be involved (47). Chen *et al.* noted that leptin did not influence glucose or GLP-1-induced insulin secretion from islets of either *ob/ob* or lean mice (47) but did attenuate basal insulin release from *ob/ob* islets by activators of protein kinase C. This observation provided evidence that leptin may additionally act during fasting to diminish insulin secretion and that this suppression is alleviated in the presence of nutrients. Thus, leptin appears to suppress both basal and glucose-stimulated

leptin secretion as evidenced by several studies that show leptin reducing insulin release from perfused pancreas and isolated islets in hypoglycemic (48, 49), euglycemic (50, 51) and hyperglycemic conditions (48, 49, 52-55).

It is worth noting that the inhibition of insulin secretion by leptin may be overridden by the incretin hormone, glucagon-like peptide-1 (GLP-1) (44), thereby assuring adequate insulin secretion in response to meals. Leptin and GLP-1 have counterregulatory roles in the regulation of both insulin secretion and biosynthesis (56). This is supported by the fact that mice in which the GLP-1 receptor was knocked out were more sensitive than wild-type animals to the insulin lowering effect of leptin (57). Similarly, rats that expressed defective leptin receptors (*fa/fa*) secreted approximately 5 times as much insulin as controls in response to GLP-1 (58). It is possible that without functional leptin receptors, the stimulatory effects of GLP-1 on insulin release are unopposed in *fa/fa* rats. Conversely in obese, hyperglycemic conditions, such as seen with diet-induced obesity, sustained elevated levels of plasma leptin may be free to signal, in particularly the fasting state, unopposed by GLP-1. Chronic leptin stimulation of insulin secretion may subsequently lead to unresponsiveness in β cells to the effects of leptin. Therefore, a loss of sensitivity to leptin by β cells could result in the deregulation of the bidirectional feedback loop between adipose tissue and pancreatic β cells resulting in the failure to suppress insulin secretion and the development of persistent hyperinsulinemia.

It has been proposed that impairment in this adipoinsular axis may also occur independently of obesity, in subjects who are susceptible to developing diabetes (59). Further investigations into the utilization of leptin in the maintenance of β cell function in populations with a genetic predisposition may prevent the onset or deter progression of obesity and diabetes.

5.3. Summary: Leptin Effects in the Liver

It is well recognized that aggressive control of hyperglycemia in patients with diabetes can delay the onset of chronic complications such as retinopathy and nephropathy (60). Current therapies for type 2 diabetes intended to reduce hyperglycemia consist of agents such as α-glucosidase inhibitors, which interfere with glucose absorption in the gut, sulphonylureas that increase insulin release from β cells, metformin which acts by reducing hepatic glucose production, and peroxisome proliferators-activated receptor-γ (PPARγ) agonists (thiazolidinediones), which enhance insulin action. Unfortunately, these therapies are limited in efficacy and give rise to side effects, one of which is obesity. Because insulin is a potent anabolic agent, the possibility of improving insulin sensitivity while maintaining appropriate weight control is a clear advantage for a drug designed with leptin-like characteristics.

The liver has a critical role in the regulation of glucose production through either *de novo* glucose synthesis or catabolism of glycogen. A relative decrease in insulin sensitivity can lead to exaggerated output of glucose from liver, which largely accounts for the development of hyperglycemia in patients with diabetes (61). In our study, we demonstrated that leptin treatment of *ob/ob* mice dramatically reduced plasma glucose concentrations, prior to changes in either food intake or body weight. An accompanying reduction in plasma insulin levels suggested improved overall insulin sensitivity. In particular, leptin improved insulin signalling through both IR and IRS-1 in liver but not in muscle or adipose tissue after 2 days of treatment. As others have documented direct actions of leptin on both muscle and adipose tissue, it is logical to believe that given a longer treatment dose, leptin would have similarly improved insulin signalling in these tissues.

The detection of leptin receptors *in vivo* in livers of mice and the presence of STAT3 signalling in FAO hepatocytes support the presence of functional leptin signalling in the liver. Furthermore, leptin activation of IR phosphorylation in HepG2 human hepatoma cells in the presence and absence of insulin

demonstrated that leptin has both insulin-mimetic and insulin-sensitizing effects. Leptin has been shown to interact with intermediates in the insulin signalling cascade including IR (62), IRS-1 and -2 (62-64), PI3K (63-66), protein kinase B (63, 65) and phosphodiesterase 3B (65, 67) and insulin reciprocally activates JAK2, STAT3 and STAT5 (68). It can be expected that enhanced insulin signalling contributes to leptin-induced glycogen synthesis and preservation of glycogen stores. An increase in insulin signalling does not, however, explain the effects of leptin on the gluconeogenic pathways since insulin is a key controller of glucose flux, suppressing hepatic glucose production and stimulating glycolysis. Leptin, in contrast, up-regulated expression of PEPCK, the rate-limiting enzyme of gluconeogenesis (62, 69-71). Synergistic activation of both insulin-dependent and -independent pathways may be required to achieve the desired physiological goal of leptin in the liver.

It is postulated that the primary role of leptin in the liver is to redistribute nutrient fluxes. Activation of gluconeogenic enzymes would utilize pyruvate as a substrate and thereby limit its availability for the Krebs cycle (72). By doing so, the liver is encouraged to oxidize fatty acids as a nutrient source and thereby limit formation of triglycerides (69, 73). Ultimately, leptin serves to protect the liver from accumulation of fat deposits when food is abundant. Therefore, although leptin promotes insulin's actions on glycogen synthesis and suppression of hepatic glucose output, leptin mediates anti-insulin effects on lipid biosynthesis, preferentially oxidizing fatty acids. Through this mechanism, leptin is capable of reversing steatosis (74, 75) while normalizing hyperglycemia. Therefore, the benefits of using leptin as a therapy in states of lipid overload surpass those of insulin sensitizers that so often cause further exacerbation of the obese state.

5.4. Summary: Contribution of PTP1B to Leptin Resistance

The rampant search for the key causative factor of leptin resistance has revealed a number of plausible candidates. For example, SOCS is an antagonist of leptin signalling that is rapidly induced upon activation of leptin signalling (76). Although increased SOCS expression in tissues has been implied in some models of diet-induced or age-related obesity (77), the role for SOCS in obesity has not yet been clarified. PTP1B, a well-established inhibitor of insulin signalling, is another candidate regulator of leptin sensitivity. PTP1B is ubiquitously expressed, including in tissues that are key regulators of insulin metabolism such as liver, muscle and fat (78). A number of non-specific protein tyrosine phosphatase inhibitors such as vanadium and peroxovanadium and their derivatives have been identified and their efficacy in restoring insulin sensitivity in patients with type 2 diabetes has been investigated (79-81). Alterations in the expression levels of various PTPs, including PTP1B, have been reported in human and rodent models of diabetes and obesity (reviewed in (82, 83)). Although there are inconsistencies in these reports, there is nonetheless a role for PTP1B in insulin resistance.

Analysis for polymorphisms in the regulatory and coding regions of the human *PTP1B* gene revealed that a mutation in the 3' untranslated regions (3'UTR) of the *PTP1B* gene leads to stabilization of its mRNA (84). Variants in 3'UTR regions have been reported to be associated with insulin resistance (85, 86). In another study, a missense variant in the *PTP1B* gene was discovered in the cDNA from a small proportion of patients with diabetes that were studied (87). An additional single nucleotide polymorphism in the PTP1B gene may confer a reduced risk of diabetes (88). We speculate that PTP1B coordinately regulates both insulin and leptin signalling to maintain the homeostatic control of body weight and metabolic parameters, that when disrupted, lead to diabetes (Fig. 5.1).

In our study, we explored the relationship between leptin, PTP1B and insulin sensitivity in mouse models of obesity and insulin resistance. Although

ob/ob mice present with exaggerated hyperinsulinemia and hyperglycemia and are clearly insulin resistant, PTP1B protein expression levels in the insulin sensitive tissues, liver, muscle and adipose tissue are reduced. This observation is in apparent contradiction to our original hypothesis that PTP1B levels is elevated and account for the impaired insulin action in these tissues. However, the recognition that leptin treatment of *ob/ob* mice increased PTP1B supports a proposed physiologic feedback loop. Similarly to the relationship that exists between leptin and SOCS (76, 89), under normal circumstances, activation of leptin signalling induces a corresponding increase in PTP1B activity. The activation of PTP1B constitutes a necessary auto-regulatory mechanism that facilitates appropriate amounts and duration of leptin signalling. Therefore, it is postulated that in the absence of leptin, the signal to induce the activation of PTP1B is missing. In the presence of leptin, as with recombinant leptin administration, leptin signalling resumes, PTP1B activity is consequently increased and the physiological response is restored.

A similar feedback system exists between insulin/insulin like growth factor (IGF-1) and PTP1B (90). Kenner *et al.* determined that incubation of rat L6 skeletal muscle cells with insulin for 12 h and IGF-1 for 24 h maximally induced PTP1B mRNA and protein (90). Enhanced expression of PTP1B mRNA and protein were associated with chronic stimulation of PTP1B activity. The mechanism by which insulin increased the catalytic activity of PTP1B is possibly through phosphorylation of PTP1B by IR kinase (91). PTP1B then dephosphorylates IR and IRS (92). Based on these observations, it is suggested that ligand-stimulated PTP1B activity desensitizes cells to the toxic effects of prolonged exposure of insulin and IGF-1. Therefore, long-term stimulation of PTP1B activity by chronic exposure with insulin may be a protective mechanism, designed to protect cells from the anabolic deposition of fat in non-adipose tissue and prevent the preferential conversion of glucose to triglycerides in liver, muscle, and heart in the presence of nutrient abundance.

Deregulation of this feedback mechanism may explain the development of leptin resistance despite higher than normal circulating leptin concentrations in human obesity. However, the evolutionary advantage of preventing leptin action is not clear and thus PTP1B-induced down-regulation of leptin signalling may be a mal-adaptive response to a plentiful nutrient energy state that was, until recently, considered to be uncommon. While numerous studies have examined the pathology of rodents and humans lacking the ability to produce or respond to leptin, little is known regarding the consequences of excessive leptin stimulation. In early parabiotic studies, fusing the circulation between *db/db* mice with +/+ mice manifested in death by starvation in the +/+ mice (93). Fully responsive to the excessive circulating concentrations of the satiety factor, now recognized as leptin, the +/+ mice had likely not developed adequate counter-regulatory mechanisms to oppose the actions of leptin. It is tempting to postulate that PTP1B and other such inhibitors of leptin action may have been designed to prevent this scenario.

While the consequences of unopposed hyperleptinemia in leptin sensitive tissues poses a potential risk of starvation, this clinical scenario nevertheless has not yet and may never, present itself in the human population. Rather, elevated leptin levels with concurrent resistance in leptin-sensitive tissues define most human obesity (19, 94). While genetic predisposition is a significant factor in the predilection for developing obesity, it is evident from population studies that abrupt changes in lifestyle and relocation to areas where available food is generally higher in dietary fat contribute substantially to obesity and diabetes (95). Increased adiposity and a subsequent rise in circulating leptin concentrations could be the primary defect that occurs with the stresses of a high fat diet. Consequently, leptin-induced elevations in PTP1B expression and activity occur, resulting in the down-regulation of the leptin signal. In this view, PTP1B acts to monitor the extent to which the signalling cascade are activated, putting a halt on the situation when it deems that further exacerbation of this pathway may present as a detriment to the cell. Inherently, a disruption in the

autoregulatory feedback loop between leptin and PTP1B would explain the progression of leptin resistance with increasing fat mass and leptin production. In these studies, the concurrent development of hyperleptinemia, hyperglycemia and hyperinsulinemia, occurred upon feeding of a high fat diet to wild-type mice. Although high fat fed mice have increased concentrations of leptin, they continue to gain weight and maintain elevated food consumption. Furthermore, high fat fed mice have reduced responses to exogenous leptin treatment, indicating the development of resistance to the satiety and metabolic actions of leptin.

The hypothesis that the abundance of leptin signalling chronically increases the expression of PTP1B and thereby maintains the phosphatase in a constitutively active state was explored. Analysis of tissues from high fat fed mice revealed that liver expressed elevated expression of PTP1B but this increase was not observed in muscle or fat. We demonstrated that leptin is an important regulator of insulin signalling in hepatocytes and thus a disruption in leptin and thereby insulin signalling by PTP1B could explain observed hyperglycemia in high fat fed mice.

It is, however, increasingly evident that dietary contents and not hyperleptinemia alone can influence the degree of leptin sensitivity. It has recently been demonstrated that transgenic mice that constitutively overexpress leptin remained extremely lean on a normal diet (96). However, when these mice were placed on a high fat diet they were no longer responsive to leptin and became obese. In this study, it was postulated that an increase in lipids, courtesy of the high fat content of the diet, interfered with leptin signalling in the hypothalamus (97). Fructose feeding also induced hepatic insulin resistance and this was attributed in part to increased expression of PTP1B in the liver (98). It has yet to be determined whether fatty acids, fructose or other nutrients can directly regulate PTP1B expression and activity. Future studies in this area may provide valuable insight into the relationship between diet and insulin and leptin resistance.

In high fructose-fed rats, hepatic insulin resistance has been associated with elevated mRNA expression levels of PTP1B and SREBP-1 (99). SREBP-1 is a key regulatory element in the development of hypertriglyceridemia since activation of SREBP-1 results in increased lipogenic gene expression, fatty acid synthesis and triglyceride accumulation. Direction of fatty acids towards lipogenesis is mediated primarily by insulin as hyperinsulinemia persistently activates SREBP-1 transcription. When PTP1B was overexpressed in hepatocytes, SREBP-1 mRNA expression was elevated to a level comparable to that seen after insulin stimulation alone (99). Based on these findings, it has been proposed that PTP1B is a crucial molecule in the pathogenesis of hypertriglyceridemia. Although, in this setting, PTP1B seems to mediate an insulin-like rather than anti-insulin effect, PTP1B may still be effectively inhibiting leptin's actions since hyperleptinema has been shown to lower hepatic SREBP-1 enzyme levels in wild-type mice (100). Therefore, PTP1B may inhibit specific leptin-regulated gene targets independently of its suppressive effects on insulin signalling.

Consistent with the idea that PTP1B is a regulator of lipid and glucose metabolism in liver, treatment of *ob/ob* mice with antisense oligonucleotide (ASO) directed against PTP1B mRNA (PTP1B ASO) resulted in downregulation of genes involved in lipogenesis in liver (101). However, expression of genes involved in gluconeogenesis, PEPCK and fructose-1,6-bisphosphatase were also downregulated in livers of *ob/ob* mice with PTP1B ASO treatment. While consistent with an inhibitory effect of PTP1B on insulin-suppression of gluconeogenesis, PTP1B ASO seemingly mimicked the effects of leptin on hepatic gene expression. However, PTP1B ASO was studied in *ob/ob* mice that do not produce leptin and although there is evidence of cross-talk between the insulin and leptin signalling pathways (68), in the absence of leptin, reduced PTP1B expression was able to enhance insulin but not leptin action. Therefore, studies in a diet-induced model of rodent obesity that is similar to human obesity

that has high circulating levels of leptin, may provide clarity in regards to the relationship between PTP1B and leptin in the liver.

Our studies demonstrate that diet-induced obese (DIO) mice respond poorly to exogenously administered leptin. We postulated that DIO mice with liver-specific suppression of PTP1B would likely have improved responses to leptin through hepatic leptin receptors and signalling pathways. Conversely, we explored the hypothesis that up-regulation of PTP1B in the liver contributes to the development of leptin resistance. As proposed, liver-specific overexpression of PTP1B in *ob/ob* mice inhibits the ability of leptin to reduce plasma glucose levels. However, leptin is still able to reduce plasma insulin concentrations, presumably because PTP1B expression is not elevated in the pancreas. Despite the absence of adenovirus-mediated up-regulation in the hypothalamus, inhibition of leptin-induced satiety was observed in *ob/ob* mice with increased hepatic PTP1B expression. One possible explanation of this finding is the existence of communication pathways amongst tissues, potentially through secreted factors. PTP1B overexpression in muscle resulted in decreased hepatic insulin-stimulated PI3K activity (102), an effect that would presumably also occur in liver-targeted PTP1B up-regulation. Sensors in the liver that detect changes in metabolism may send communicating signals to the brain to modify food intake. If this situation holds true, peripheral as well as central leptin sensitivity may be influential in feeding behavior and possibly thermogenic regulation.

Despite our observation that PTP1B is preferentially up-regulated in livers of high fat fed mice in the time frame that we studied, elevated levels of PTP1B in muscle and fat could also contribute to leptin and insulin resistance. Mice with a disruption of the PTP1B gene ($PTP1B^{-/-}$), maintained lowered glucose concentrations despite reduced circulating concentrations of insulin, implying an increase in insulin sensitivity (103, 104). In response to administration of a bolus of glucose, more rapid clearance of glucose was observed in $PTP1B^{-/-}$ mice in comparison to wild-type mice. This improvement in glucose turnover was partially attributed to increased IR and IRS-1 phosphorylation in response to

insulin in muscle tissues of PTP1B$^{-/-}$ mice (103). Muscle specific overexpression of PTP1B in mice resulted in decreased insulin-stimulated glucose uptake specifically in muscle and caused whole body insulin resistance (102)

Interestingly, an improvement in insulin sensitivity in PTP1B$^{-/-}$ mice did not apply to white adipose tissue (104), which may explain their leanness, decreased white adipose stores and reduced propensity for developing obesity (104). Increased insulin signalling in adipose tissue promotes adipocyte differentiation and upregulation of the genes involved in lipogenesis. However, in PTP1B$^{-/-}$ mice compared to wild-type controls, there was a noticeable reduction in fat cell mass without a decrease in adipocyte number (104). This leaves the possibility that perhaps PTP1B inhibition in adipose tissue preferentially enhanced the lipolytic effects of leptin. PTP1B ASO treatment of *ob/ob* mice resulted in downregulation of genes in adipose tissue that are involved in lipogenesis and adipocyte differentiation. The regulation of many of the genes in adipose tissue with PTP1B ASO treatment caused an opposite reaction than that which occurs with treatment with the insulin-sensitizing class of drugs known as thiazolidinediones (TZD) which have been shown to cause adipocyte differentiation and upregulation of genes involved in lipogenesis (105-107). With TZD treatment, more fat cells of smaller average size were produced which are more sensitive to insulin-dependent glucose uptake (reviewed in (108)). Thus, an increase in weight gain is expected with TZD treatment and has been confirmed in clinical studies (108).

In contrast, *ob/ob* mice treated with an antagonist to PTP1B displayed resistence towards increasing adipose mass when placed on a high fat diet (101). PTP1B ASO treatment decreased lipogenesis in WAT, downregulating several genes involved in triglyceride synthesis and including peroxisome proliferators-activated receptor γ (PPARγ), a member of the nuclear hormone receptor family of ligand-activated transcription factors that plays a key role in fat cell differentiation (109). Interestingly, leptin treatment also resulted in a similar

downregulation of genes involved in lipogenesis (110, 111), suggesting that inhibition of PTP1B facilitates improved leptin signalling.

PTP1B ASO treatment was studied in *ob/ob* mice that do not produce leptin. It is possible that reductions in PTP1B activity primes the leptin signalling pathway by sensitizing components of the leptin signalling pathway in preparation for ligand binding. Through crosstalk with either insulin or an unidentified molecule, signalling via JAK/STAT is thereby activated in the absence of leptin. Alternatively, both leptin and PTP1B may modulate some of the same pathways pertaining to glucose and fatty acid metabolism. Cheng *et al.* demonstrated that the loss of PTP1B from *ob/ob* mice resulted in reduced weight gain and body fat in comparison to *ob/ob* mice with PTP1B intact, implying that PTP1B alone can regulate obesity in a leptin-independent manner (112). However, exogenous administration of leptin to mice with a PTP1B deficiency improved leptin sensitivity. Thus PTP1B regulates body weight and likely lipid metabolism in adipocytes via both leptin-independent and dependent pathways. Nevertheless, the selectivity of PTP1B antagonists for promotion of leptin-like signalling in some tissues and insulin-sensitizing effects in others makes this class of compounds ideal in the treatment of both obesity and diabetes.

Improved pancreatic β cell function may be an additional advantage for the development of PTP1B inhibitors. In IRS2 deficient mice ($IRS2^{-/-}$), which have diabetes attributed to β cell failure and not insulin resistance, the superimposition of a PTP1B disruption partially restored β cell function (113). $IRS2^{-/-}$ mice displayed progressive deterioration of glucose homeostasis due to lack of β cell compensation for insulin resistance in liver and skeletal muscle (114). PTP1B disruption in $IRS2^{-/-}$ mice promoted islet growth and function, as evident by a reduction in islet size and cross-sectional β cell area and increased insulin immunostaining β cells. Since failure of pancreatic β cells to produce sufficient insulin fuels the deterioration of glucose control, pharmacological intervention with PTP1B antagonists may preserve β cell function and delay the onset of diabetes.

5.5. Future Research

5.5.1. *Further evaluation of contribution of liver and pancreatic β cells in leptin-regulated glucose and lipid homeostasis*

The results of the studies described in this thesis, together with previous studies on the peripheral endocrine role of leptin, indicate that leptin may have a significant impact on glucose homeostatic regulation via signalling pathways in the liver and pancreatic β cells. Some designs for future research to determine the relative contribution of these non-central organs to the regulation of insulin and glucose tolerance include the following: (1) **targeting the *ObRb* gene to hepatocytes of *db/db* mice by adenoviral gene delivery**. Administration of adenoviral vectors via tail vein injections is an efficient means by which various genes can be delivered to hepatocytes. Normally, *db/db* mice are unresponsive to the actions of exogenously administered leptin because of the absence of functional ObRb. The introduction of adenovirus carrying the *ObRb* gene in adult *db/db* mice will permit liver-specific leptin signalling. Upon administration of leptin to these mice, it is anticipated that any observed changes can be attributed to hepatic leptin signalling. (2) **selective deletion of *ObRb* gene at different stages of development by utilizing a Cre-loxP system in which an albumin or insulin promoter is used to direct tissue-specific expression of Cre recombinase in hepatocytes and β cells, respectively.** The Cre-loxP system consists of the generation of two lines of transgenic mice (115-117). One line of mice contains the *cre* gene, which encodes a site-specific DNA recombinase called Cre. The other line of mice has a *loxP* sequence in their genome which acts as flags, identifying sequences for Cre recombinase to act upon. Breeding of these two lines of mice results in mice whose cells have *loxP* sites and also express Cre. The loxP sites are recognized by Cre, resulting in excision of DNA between the loxP sites and subsequent re-ligation by Cre. As a result, a specific sequence of DNA can be removed from the genome. Tissue specificity is achieved by having the *cre* gene bound to a tissue-specific promoter such as albumin in the liver and insulin in β cells. Cohen *et al.* used a Cre-loxP strategy

to create mice with neuron-specific deletion of ObRb (118). While these mice became obese, the weight gain was not as profound as was observed in ObR-null mice. This observation suggested that leptin has effects on the brain but did not exclude the possibility that leptin also has direct actions in the periphery. When they looked at mice with hepatocyte-specific deletion of leptin receptors, the absence of hepatic ObRb had no effect on weight distribution, lean or fat mass, plasma insulin, glucose or leptin concentrations or liver triglyceride content in comparison to control mice. It was concluded from this study that the liver abnormalities observed in *ob/ob* mice and *db/db* mice are secondary to defective leptin signalling in the brain. Because these mice are defective in hepatic leptin signalling from birth, it is uncertain whether mechanisms mediated centrally or via other peripheral tissues might compensate for this deficiency during development and thereby mask early phenotypic changes. We propose that a more effective strategy would be the utilization of an inducible Cre model in which the expression of the ObRb gene can be selectively down-regulated at different stages of development. The combination of the Cre/*lox* and tetracycline-regulated transcriptional systems allows for expression of Cre only when the drug tetracycline is administered (119). Acute disruptions in leptin signalling in a liver or β cell specific manner may reveal pathways that are immediately disrupted in the absence of functional leptin signalling. It is anticipated that these studies will further our understanding of leptin-mediated actions in non-central tissues and its relative contribution to glucose and lipid homeostasis.

5.5.2. *Future studies evaluating the mechanisms by which PTP1B inhibits leptin signalling*

In our studies, we have determined that PTP1B is a critical regulator of both the insulin and leptin signalling pathways. Although previous studies have demonstrated inhibition of JAK2 phosphorylation by PTP1B (112, 120) and we have determined that PTP1B down-regulates STAT3 phosphorylation, further understanding of the effect of PTP1B on leptin signalling cascade may provide

additional insight into this relationship: ***Analysis of the direct effect of PTP1B on STAT and ObRb phosphorylation.*** Although we observed that PTP1B reduces STAT3 phosphorylation, it is not known whether it is a direct inhibition of the phosphatase on the STAT3 molecule or if this effect is a downstream consequence of JAK2 inhibition. To further clarify this mechanism, it would be of interest to induce the activation of STAT3 and ObRb by treating ObRb-expressing cells with leptin, then immunoprecipitate out STAT3 and ObRb and co-incubate with PTP1B to determine whether PTP1B can specifically inhibit these two targets. Another mechanism is the use of fluorescence resonance energy transfer (FRET) to detect interactions between PTP1B with STAT3 and PTP1B with ObRb (121). FRET would involve the attachment of fluorescent molecules to the proteins and visualizing the emission signals from the cells upon leptin stimulation. Computational analysis would provide information regarding spatial and temporal interactions of molecules in both inactivated and activated states. These proposed studies may provide additional insight into the mechanisms by which PTP1B inhibits the leptin signalling pathway.

5.5.3. *Future studies exploring the relationship between high fat diets, PTP1B and leptin and insulin resistance*

We established that a correlation between high fat feeding and PTP1B expression exists in DIO mice. Strategically, identification of the causes of elevated PTP1B may be beneficial in preventing the onset of insulin and leptin resistance. Results from the following studies may provide additional insight into the regulation of expression and activity of PTP1B and further evaluate the role of PTP1B in insulin and leptin sensitivity: (1) ***In vitro*** **analysis of the effects of fatty acids and variability in glucose concentrations on PTP1B expression.** In addition to insulin, we have shown that leptin can also up-regulate PTP1B expression. Whether certain nutrients derived from high-caloric diets or other factors secreted from increasing adipocyte mass can also induce changes in PTP1B expression is yet to be determined. It would be of interest to evaluate the

effects of various fatty acids and different concentrations of glucose on PTP1B expression in hepatocyte cultures. (2) **Evaluation of the effects of down-regulating PTP1B on downstream insulin and leptin signalling pathways.** The application of a PTP1B antagonist might help to identify pathways that are normally down-regulated in the presence of elevated phosphatase activity. Initial *in vitro* studies may be performed in which the PTP1B antagonist is administered prior to or with leptin treatment of cells. Assessment of JAK2 or STAT3 phosphorylation of STAT3 nuclear translocation would determine whether the PTP1B antagonist could effectively down-regulate leptin signalling. Once and inhibitory effect of the antagonist is established, it would be of interest to evaluate the potential of the antagonist in the treatment of obesity and diabetes in DIO mice and determine anticipated efficacy and side-effects when translated to clinical applications.

5.6. Conclusion

Classically, leptin has been viewed to mediate its actions primarily in the central nervous system. These studies provide support that interactions with leptin receptors in non-central tissues, in particular, pancreatic β cells and liver, also activate essential pathways that are critical in the monitoring and regulation of nutrient fluxes. In β cells, leptin reduced insulin secretion, which may contribute to the normalization of hyperinsulinemia that is observed following leptin treatment of *ob/ob* mice. In the liver, leptin significantly improved insulin signalling, a finding consistent with suppression of hepatic glucose production and reductions in fasting plasma glucose concentrations in *ob/ob* mice when leptin is given *in vivo*. We established that disruptions in a normal physiological pathway that down-regulates insulin and leptin signalling may eventually result in the pathology of insulin and leptin resistance, respectively. In light of these findings, we speculate that leptin is a link between obesity and diabetes and that disruptions in leptin signalling may contribute to pathophysiology of both diseases (Fig. 5.2). The consumption of lipid rich foods in today's society results

in insulin-directed storage of triglycerides and expansion of adipocyte mass. In response, numerous adipocytokines including leptin are released into the circulation in direct proportion to adiposity. Initially cells are responsive to leptin, and leptin capably protects non-adipocytes from the toxic effects of excessive amounts of circulating lipids, potentially through the activation of PTP1B-mediated down-regulation of insulin signalling in specific tissues including liver and skeletal muscle but excluding adipose. Eventually, perhaps when leptin concentrations surpass a certain threshold, persistent stimulation of PTP1B by leptin precipitates both insulin and leptin resistance through the inhibitory effects of PTP1B on the signalling pathways of both hormones. This response alters the effects of leptin on other physiological systems and thus leads to impaired regulation of appetite, thermogenesis, reproduction, immune function, and glucose and lipid homeostasis. It is postulated that interruption of this counterproductive exacerbation of the normal regulatory inhibition of leptin signalling may prevent diabetes perpetuated by obesity. Thus leptin may be the key determinant factor linking these two diseases and it is anticipated that further understanding of the role of PTP1B in the development of leptin and insulin resistance will manifest in exciting new therapeutic strategies targeted at both obesity and diabetes.

5.7. References

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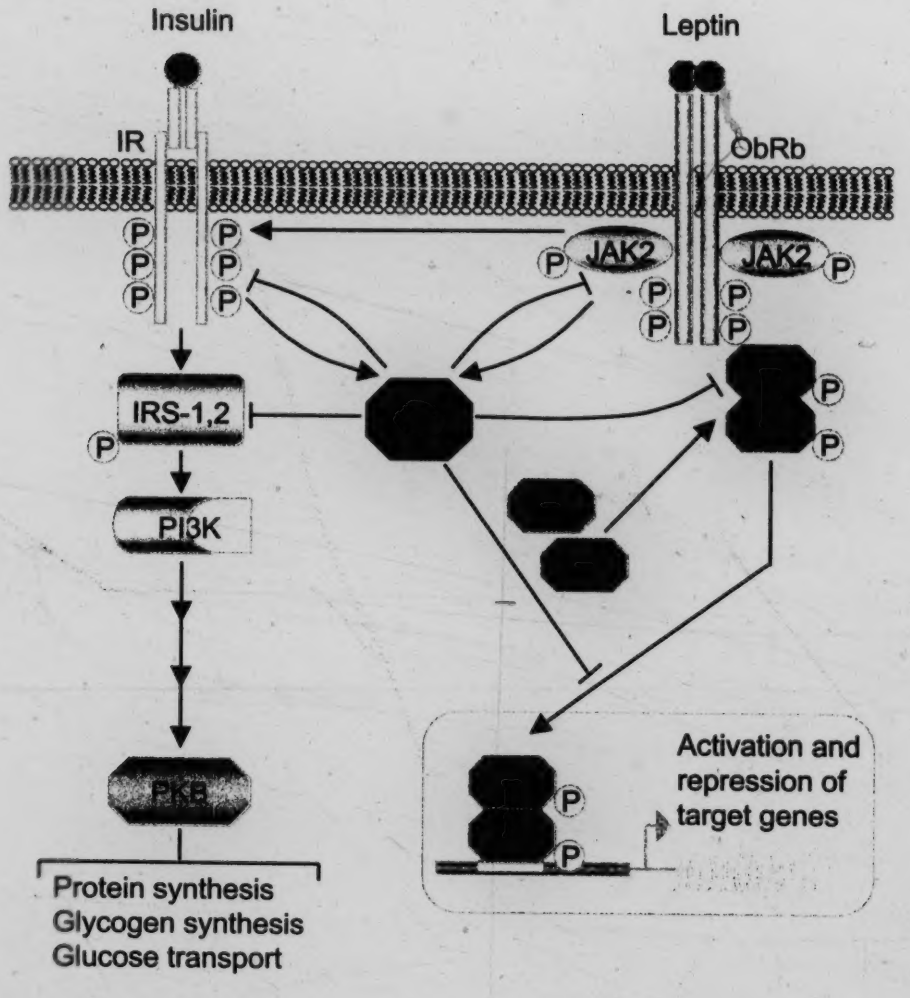

Figure 5.1. Interaction of PTP1B with leptin and insulin signalling. In our studies, we demonstrated that leptin induces IR phosphorylation. IRS-1 phosphorylation is also activated by leptin although it is unknown whether the mechanism is direct or a consequence of IR phoshorylation. Leptin also increases PTP1B expression and we postulate that activation of PTP1B by insulin and leptin serves as a negative regulatory feedback mechanism to maintain appropriate levels of receptor signalling. PTP1B decreases insulin signalling via dephosphorylation of IR and IRS-1. PTP1B reduces phosphorylation of JAK2 and STAT and inhibits nuclear translocation of STAT, thereby reducing leptin signalling. It is uncertain whether the effects of PTP1B on STAT phosphorylation and translocation are direct or downstream of JAK2. Given that PTP1B downregulates both insulin and leptin signalling, inappropriate levels of PTP1B activity may consequently precipitate both insulin and leptin resistance.

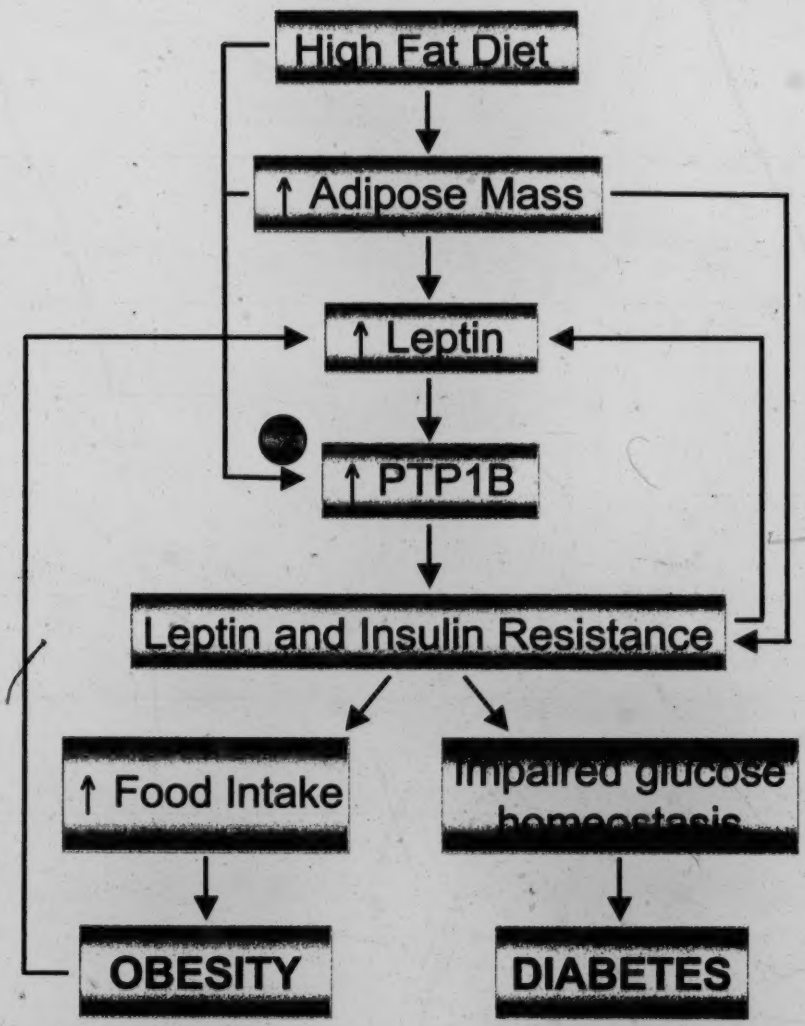

Figure 5.2. Proposed model for the pathogenesis of obesity and diabetes. Consumption of a diet consisting of high fat foods results in the storage of fatty acids as triglycerides and the expansion of adipocyte mass. Increasing amounts of leptin and other adipocyte-derived factors are secreted. Our studies show that leptin can increase PTP1B expression in liver and skeletal muscle and we propose that PTP1B expression is possibly also elevated in the hypothalamus and other tissues. Besides leptin, other factors including nutrients from the diet and adipocytokines may also cause an elevation in PTP1B expression. PTP1B downregulates leptin and insulin signalling, leading to resistance to the actions of both hormones. Other fat-derived factors like fatty acids and tumor-necrosis factor α may also contribute to leptin and insulin resistance. Leptin and insulin resistance results in inappropriate regulation of food consumption and metabolic parameters of glucose metabolism. Obesity further exacerbates leptin and insulin resistance, perpetuating the disease states of obesity and diabetes.